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ORIGINAL ARTICLE

Isolation, selection and evaluation of antagonistic yeasts and lactic acid bacteria against ochratoxigenic fungus *Aspergillus westerdijkiae* on coffee beans

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Significance and Impact of the Study: Studies based on microbial ecology and antagonistic interactions are important for the development of new strategies in controlling aflatoxin contamination of crops and are relevant to further biotechnological applications. This study shows that coffee fruit is a potential source for the isolation of microbial strains with antifungal ability. A new yeast strain, *Pichia fermentans* LPBYB13, showed efficacy in reducing growth and ochratoxin A production of *Aspergillus westerdijkiae* in coffee beans. Our results should encourage the use of this yeast strain on a large scale for biocontrol of aflatoxigenic fungi in coffee beans.

Keywords
coffee processing, fungistatic action, *Lactobacillus*, Ochratoxin A, *Pichia fermentans*.

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Abstract

In this study, yeasts and lactic acid bacteria (LAB) were isolated from coffee fruits and identified via biochemical and molecular approaches. The isolates represented the *Pichia*, *Debaryomyces*, *Candida*, *Clavispora*, *Yarrowia*, *Sporobolomyces*, *Klyveromyces*, *Torulaspora* and *Lactobacillus* genera. Four isolates, namely *Pichia fermentans* LPBYB13, *Sporobolomyces roseus* LPBY7E, *Candida* sp. LPBY11B and *Lactobacillus brevis* LPBB03, were found to have the greatest antagonist activity against an ochratoxigenic strain of *Aspergillus westerdijkiae* on agar tests and were selected for further characterization. Applications of *P. fermentans* LPBYB13 in coffee cherries artificially contaminated with *A. westerdijkiae* showed efficacy in reducing ochratoxin A (OTA) content up to 88%. These results highlight that *P. fermentans* LPBYB13 fulfils the principle requirements of an efficient biological control of aflatoxigenic fungi in coffee beans and may be seen as a reliable candidate for further validation in field conditions.

Introduction

Coffee has been for decades the most commercialized food product and most widely consumed beverage in the world, with over 600 billion cups served per year. Before coffee cherries can be traded and processed into a final industrial product, they have to undergo postharvest processing on farms, which have a direct impact on the cost and quality of coffee (Pereira *et al.* 2015a). Several species of fungi grow in coffee cherries during processing and are associated with poor beverage quality (negative flavour modifications) and the production of mycotoxins potentially harmful to consumers, mainly ochratoxin A (OTA) (Nakajima *et al.* 1997; Iamanaka *et al.* 2011). *Aspergillus westerdijkiae* is a potent OTA producer that has been found in coffee beans (Mata *et al.* 2007). This mycotoxin remains bioactive through the final stages of coffee production and consumption (i.e. the toxin is not destroyed in roasting or brewing). Thus, several studies have reported the incidence of OTA in roasted coffee beans exported to consumer countries, such as Switzerland (Studer-Rohr *et al.* 1995), Spain (Burdaspal and Legarda 1998) and England (Patel *et al.* 1997).

OTA is known to have nephrotoxic effects and carcinogenic potential in animal species (Mata *et al.* 2007). With the exception of Italy, which in 1996 set a limit of 4 ng g^{-1}

of OTA, the other countries of the European Union have no regulations for this toxin. However, since 2002, countries such as Spain, Italy and Holland are evaluating the safety and quality of coffee by introducing mycotoxin quantification. The use of fungicides is widely employed to reduce aflatoxin accumulation in agricultural products during field processing. However, the use of these chemical can cause adverse effects on consumers and environment, and may lead to extreme cases of acute and chronic toxicity. Nowadays, biological control methods are broadly used for the management of many phytopathogenic fungi (Zucchi *et al.* 2008). On this account a considerable number of formulations, using a large range of yeast and bacteria species, have already been used commercially (Cole and Cox 1981; Northolt *et al.* 1995; Petersson and Schnürer 1995; Paster *et al.* 1997). Some possible mechanisms behind antagonist activity are currently suggested, such as competition for nutrients and space, adhesion of the antagonist cells to the mycelium of the fungi and/or production of extra-cellular metabolites toxic (Liu *et al.* 2013).

Yeasts and lactic acid bacteria (LAB) are considered safe micro-organisms (classified as 'generally recognized as safe'; GRAS) because the occurrence of pathogenic species in foods is practically unknown. The sugars present in coffee cherries are used by a vast variety of yeast and LAB species resulting in a number metabolites with antimicrobial activity (Studer-Rohr *et al.* 1995; Pereira *et al.* 2014). The aim of the present work was to isolate and select antagonistic yeasts and LAB against ochratoxigenic fungus *A. westerdijkiae* and to evaluate their ability to restrict OTA accumulation in coffee beans.

Results and discussion

Identification and screening of yeasts and LAB

A total of 27 yeasts and 20 LAB were isolated from coffee fruits. Based on phenotypic characteristics, biochemical tests and sequence analysis of rRNA genes, the following species were identified: *Lactobacillus* sp. (11), *Lactobacillus brevis* (5), *Lactobacillus plantarum* (3), and *Lactobacillus paracasei* (1) in the LAB group; and *Pichia anomala* (3), *Pichia fermentans* (3), *Debaryomices hansenii* (1), *Candida* sp. (2), *Candida glabrata* (3), *Candida zeylanoides* (3), *Candida maris* (2), *Candida inconspicua* (2), *Candida magnolia* (1), *Clavispora lusitaniae* (2), *Yarrowia lipolytica* (2), *S. roseus* (1), *Klyveromyces marxicianus* (1) and *Torulaspora delbrieckui* (1) in the yeast group (Fig. 1). These yeast and LAB species have been previously found in coffee processing environments (Silva *et al.* 2000; Vilela *et al.* 2010; Leong *et al.* 2014; Pereira *et al.* 2014), except for *Lact. paracasei*, *Ca. zeylanoides*, *Ca. maris*, *Ca. magnolia*, *Cl. lusitaniae* and *S. roseus*, which were for the first time isolated.

A preliminary screening was carried out with all yeast and LAB isolates to test their capability to inhibit the growth of *A. westerdijkiae*. Most isolates showed antagonistic activity on mycelial growth of the toxigenic fungus (Fig. 1). This suggested that coffee fruit is a potential source for the isolation of microbial strains with antifungal ability. Four isolates, namely *P. fermentans* LPBYB13, *S. roseus* LPBY7E, *Candida* sp. LPBY11B and *Lact. brevis* LPBB03, were found to have the highest percentages of inhibition against *A. westerdijkiae* (over 50% of inhibition) and selected for further characterization. Similar results were obtained for the effect of other yeasts (e.g. *P. anomala*, *Pichia kluyveri* and *Hanseniaspora uvarum*) against spore germination of toxigenic *Aspergillus carbonarius* or *Aspergillus ochraceus* (Masoud and Kaltoft 2006; Djossou *et al.* 2011; Leong *et al.* 2014).

Inhibitory effect of selected yeast and LAB strains against *A. westerdijkiae* was assessed through agar overlay technique at different pH values (Table 1). In this assay, *P. fermentans* LPBYB13 and *Lact. brevis* LPBB03 were able to generate an inhibitory halo against *A. westerdijkiae*, with greater effect observed for the yeast strain (inhibitory halo >0·5 cm). *Pichia fermentans* LPBYB13 performance was unaffected by pH changes while *Lact. brevis* LPBB03 loses its antagonist activity at pH 7. The physical and chemical changes that occur during coffee processing, such as environmental acidification during fermentation step, can affect performance of the selected antagonist strain (Pereira *et al.* 2014). Thus, the antagonistic ability of *P. fermentans* LPBYB13 at different pH values might be considered an advantage of this yeast strain, which would allow its action at the different stages of coffee processing.

Interestingly, no inhibition zone was observed by the cultivation of *A. westerdijkiae* with suspensions of *S. roseus* LPBY7E and *Candida* sp. LPBY11B (Table 1), although these strains presented antagonistic activity against this fungus by mycelial growth inhibition assay (Fig. 1). These findings suggest that the antagonistic action of these strains against mycelial growth of *A. westerdijkiae* may be due to nutrient competition, rather than the secretion of inhibitory substances.

The influence of *Pichia fermentans* on aflatoxin accumulation study

Co-culturing micro-organisms can result in either stimulation or inhibition of mycotoxin production (Petersson *et al.* 1998; Masoud and Kaltoft 2006). In this study, the OTA content produced by *A. westerdijkiae* on coffee beans when co-cultured with *P. fermentans* LPBYB13 and *Lact. brevis* LPBB03 was determined (Table 2). A stimulation effect of mycotoxin production was observed when *A. westerdijkiae* was co-cultured with *Lact. brevis* LPBB03.

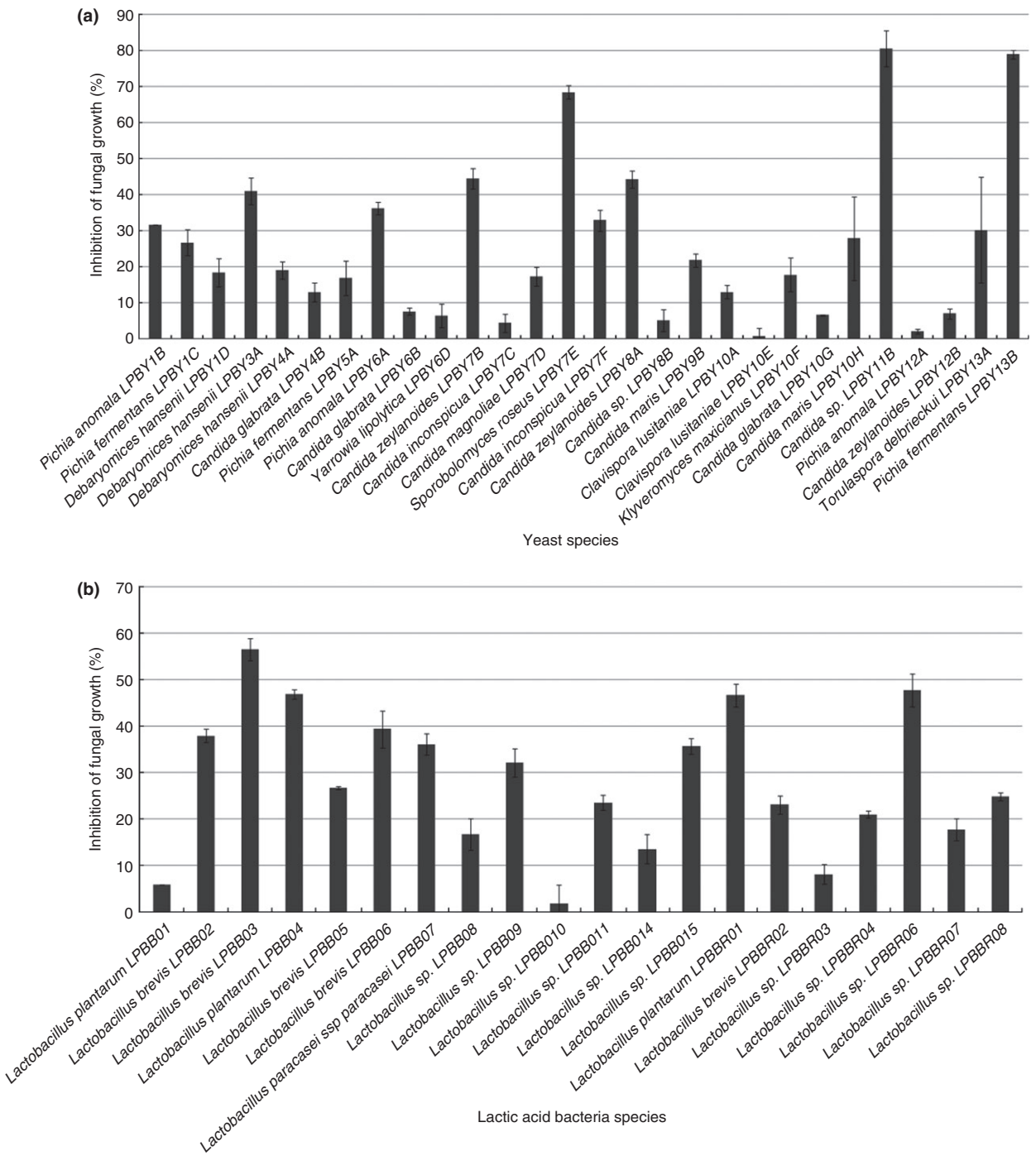

Figure 1 Percentage of inhibitory effect on *Aspergillus westerdijkiae* growth by the micro-organisms isolated from coffee cherries. (a) Yeasts. (b) Lactic acid bacteria.

On the other hand, the aflatoxin accumulation by *A. westerdijkiae* decreased drastically due to the *P. fermentans* LPBYB13 biocontrol treatment (from 6·54 to 0·76 ng g^{-1}). Thus, the reduction of the fungal viability by *P. fermentans* LPBYB13 (Fig. 1 and Table 1) was reflected on aflatoxin production and accumulation in coffee beans. In addition, reduction of OTA might also have been due its degradation or adsorption by *P. fermentans* metabolism. It has been reported that *Saccharomyces cerevisiae* and *Saccharomyces bayanus* adsorbed about 45% of OTA present in synthetic grape juice medium (Bejaoui *et al.* 2004).

Table 1 Inhibitory effect of selected yeast and LAB strains against *Aspergillus westerdijkiae* by agar overlay technique at different pH values

Selected strains	Clear zone inhibition diameter (cm)		
	pH 5	pH 6	pH 7
Pichia fermentans LPBYB13	0·55 ± 0·03	0·52 ± 0·02	0·52 ± 0·03
Sporobolomyces roseus LPBY7E	ND	ND	ND
Candida sp. LPBY11B	ND	ND	ND
Lactobacillus brevis LPBB03	0·10 ± 0·03	0·02 ± 0·01	ND

ND, not detected.

Table 2 Reductions of ochratoxin A production by *Aspergillus westerdijkiae* on green coffee beans when co-inoculated with *Pichia fermentans* LPBYB13 or *Lactobacillus brevis* LPBB03

Treatment	Ochratoxin A ng g^{-1}
C1: Uninoculated coffee beans	<0·25[a]
C2: C1 + *P. fermentans* LPBYB13	<0·25[a]
C3: C1 + *Lact. brevis* LPBB03	<0·25[a]
C4: C1 + *A. westerdijkiae* (10^6 spores g^{-1})	6·54 ± 0·37[b]
T1: C4 + *P. fermentans* LPBYB13	0·76 ± 0·09[c]
T2: C4 + *Lact. brevis* LPBB03	7·11 ± 0·57[b]

Means of triplicate in each column bearing the same letters are not significantly different ($P > 0·05$) from one another using Duncan's Test (mean ± standard variation).

The influence of the inoculum level of *P. fermentans* on OTA accumulation was tested. The results presented in Table 3 indicate that application at concentrations higher than 10^6 CFU g^{-1} is needed to reduce the OTA content from 6·54 to levels below 1 ng g^{-1}, which represents a reduction of approx. 88% compared to control (i.e. coffee beans inoculated only with *A. westerdijkiae*).

In summary, this study demonstrated the suppressive effects of *P. fermentans* LPBYB13 to reduce growth of *A. westerdijkiae* and prevent production of OTA in coffee beans. Some yeast and LAB species isolated from coffee processing has been evaluated for their antagonistic activity against toxigenic fungi (Masoud *et al.* 2005; Masoud and Kaltoft 2006; Djossou *et al.* 2011; Leong *et al.* 2014). Despite these efforts, yeast formulations are yet not applied in the coffee postharvest chain, mostly due to its inviability use in field conditions. Recently, we have demonstrated that the metabolic activity of *P. fermentans* yeast in coffee processing influences the final volatile fraction of roasted beans and promotes quality development of coffee product (Pereira *et al.* 2014; Soccol *et al.* 2013; Pereira *et al.* 2015b). All these features make its application even more interesting, leading to products of improved quality and offering consumer protection against the presence of toxic and otherwise undesirable compounds. Our results should encourage the use of *P. fermentans* on a large scale for biocontrol of aflatoxigenic fungi. More studies including field trials are now in progress.

Table 3 Reduction of ochratoxin A production by *Aspergillus westerdijkiae* on coffee beans when co-inoculated with *Pichia fermentans* LPBYB13 in different concentrations

Treatment	Ochratoxin A ng g^{-1}
C1: Uninoculated green coffee beans	<0·25[a]
C2: C1 + *A. westerdijkiae* (10^6 spores g^{-1})	6·54 ± 0·30[b]
T1: C2 + *P. fermentans* LPBYB13 (10^3 CFU g^{-1})	3·28 ± 0·09[c]
T2: C2 + *P. fermentans* LPBYB13 (10^4 CFU g^{-1})	1·84 ± 0·09[d]
T3: C2 + *P. fermentans* LPBYB13 (10^5 CFU g^{-1})	1·44 ± 0·05[e]
T4: C2 + *P. fermentans* LPBYB13 (10^6 CFU g^{-1})	0·76 ± 0·08[f]

Means of triplicate in each column bearing the same letters are not significantly different ($P > 0·05$) from one another using Duncan's Test (mean ± standard variation).

Material and methods

Isolation and identification of yeast and LAB

Twenty-five grams of coffee cherries were manually depulped, to obtain beans with mucilage. The mucilaginous parchment beans were submersed in 100 ml sterile water and placed to ferment naturally for 24 h at 28°C. Ten fold dilutions from this culture were prepared and 0·1 ml of each dilution was spread on MRS (Merck, Whitehouse Station, NJ) and DRBC (Oxoid, São Paulo, Brazil) agar plates for isolation of LAB and yeast, respectively. After spreading, the plates were incubated at 30°C for 3–4 days. Isolates were purified and stored at −80°C in 20% glycerol.

Yeast strains were physiologically characterized by determination of their morphology, spore formation and fermentation of different carbon sources, as previously described (Kurtzman *et al.* 2011). LAB strains were characterized by gram staining in conjunction with microscopic examination, determination of catalase and oxidase activities, motility tests and fermentation of different carbon sources using the API 50 CHL test strips (Biomérieux, São Paulo, Brazil).

LAB and yeast strains were identified by 16S rRNA gene and ITS region sequencing, respectively. DNA was extracted using QIAamp DNA Mini Kit (Qiagen, São Paulo, Brazil) according to the manufacturer's instructions. PCR amplification of gene was performed using the primers for 16S rDNA (27f-AGAGTT TGATCCTGGCTCAG and 1512r-CGGCTACCTTGTTACGACT) or the primers for ITS (ITS3-GCATCGATGAAGAACGCAGC and ITS4-TCCTCCGCTTATTGATATGC) (Lott *et al.* 1993; Barszczewski and Robak 2004; Wang *et al.* 2006). The PCR were gel purified using the Qiaex II Gel Extraction Kit (Qiagen, São Paulo, Brazil) according to the manufacturer's instruc-

tions. Fragments were analysed using an automatic DNA sequencer (ABI Prism 3730 XL genetic analyzer; Applied Biosystems, Foster City, CA) and queried against NCBI/GenBank databases. A percent similarity of ≥98% between the unknown sequence and the closest matching sequence from the GenBank database was used as the criterion to classify an isolate to the species level.

Screening for microbial antagonists

The antagonistic activity of yeast and LAB isolates against *A. westerdijkiae* was conducted in two steps. Firstly, 1 ml of 10^6 UFC ml^{-1} of each microbial isolate was streaked on Petri dishes containing YM (for yeast isolates) and MRS (for bacterial isolates). After homogenization and agar solidification, 0·1 ml of 10^6 spore suspension of *A. westerdijkiae* per millilitre was inoculated in the centre on the Petri dish and incubated at 28°C for 5 days. The experiment was arranged in a randomized complete design with three replications. Radial growth reduction was calculated as follows: % inhibitory effect on *A. westerdijkiae* growth = $((a - b) \times 100)/a$; where a = radial growth measurement of the pathogen in control and b = radial growth of the pathogen in the presence of micro-organism tested.

Next, the four strains with high biocontrol efficacies (*viz.*, *P. fermentans* LPBYB13, *S. roseus* LPBY7E, *Candida* sp. LPBY11B and *Lact. brevis* LPBB03) were evaluated by using the agar overlay technique at different pH ranges. 0·1 ml of 10^6 spore suspension of *A. westerdijkiae* per millilitre was poured into Petri dishes containing YM (for yeast isolates) and MRS (Merck) (for bacterial isolates). To evaluated the influence of pH on the fungistatic effect of the strains, culture media were previously adjusted to pH 5·0, 6·0 and 7·0 with 1 N NaOH or 1 N HCL. After homogenization and agar solidification, 0·1 ml of the supernatant from the yeasts and LAB cultivation were inoculated in the centre prepared with hole of a radius of 0·25 cm on the agar in the Petri dish. The inhibition zone was measured after incubation at 28°C for 5 days. Each treatment (each isolate) was done in triplicate and each experiment was conducted three times. Controls sets were simultaneously run without the cultures.

Biological control in coffee beans

The ability of the selected micro-organisms to restrict OTA accumulation was studied by inoculating 25 g of coffee beans arranged in sieves with 1 ml g^{-1} of spore suspension (10^6 spore g^{-1}) of *A. westerdijkiae*, and *P. fermentans* LPBYB13 or *Lact. brevis* LPBB03 at concentrations of 10^6 CFU g^{-1} respectively. As a control, coffee beans inoculated only with spore suspension of *A. westerdijkiae* was carried out. The coffee beans were placed in a large glass flask sealed with a cotton plug for 10 days at 28°C with humidification maintained by water in the bottom. The initial aw (water activity) before inoculation was 0·63, after 10 days it was 0·97. The quantitative analysis of OTA produced after 10 day fermentation was done as shown below. Finally, the effect of different concentrations of *P. fermentans* LPBYB13 (10^3, 10^4 or 10^5, 10^6 CFU g^{-1}) was also evaluated under conditions above mentioned due its efficacy in reducing OTA content in coffee beans. All the experiments were performed in triplicate.

Ochratoxin A quantification in coffee beans after biological control treatment

After solvent extraction and purification steps employing the Ochratest affinity column, 200 μl of each sample was injected into a high-performance liquid chromatography unit with a fluorescence detector (Agilent model 1100, Waldbronn, Germany). The chromatographic column used was an Octadecil Silan C1 (250 × 4·6 nm, with 5 μm particles) in reverse phase at 25°C; the mobile phase was acetonitrile/methanol/aqueous glacial acetic acid (1 : 1 : 0·5) at a flow rate of 1 ml min^{-1}. The injection volume was 20 μl and ochratoxin A from Sigma (São Paulo, Brazil) was used as a standard.

Statistical analyses

The data were analysed in a completely randomized design with three replicates. A Duncan's test was performed using SAS program (Statistical Analysis System, Cary, NC). Level of significance was established in a two-sided P-value <0·05.

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Conflict of Interest

The authors declared no interest of conflicts.

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