LWT - Food Science and Technology 164 (2022) 113684

Contents lists available at ScienceDirect

LWT

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

A new methodology for the analysis of total deoxynivalenol, dissolved and adsorbed on cell walls, in microbiological culture assays

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ARTICLE INFO

Keywords:
Deoxynivalenol analysis
Microbiological culture
Bacteria
Adsorption
Biodegradation

ABSTRACT

Deoxynivalenol (DON) is a mycotoxin mainly produced by *Fusarium graminearum* and *Fusarium culmorum* and is commonly found in cereals such as wheat, barley, oats, and their derivatives. Scientists have been working on different strategies for DON detoxification, with biological detoxification being an approach with growing interest. When evaluating the use of microorganisms for mycotoxin detoxification, different phenomena can occur, namely, biotransformation (by microorganism metabolism and by interaction with extracellular cell proteins) and adsorption on cell walls, both of which can be present. In this study, a fast, simple, reliable, and inexpensive method for total DON quantification (dissolved and adsorbed) in bacterial culture assays is presented. This method can be used in screenings designed for searching DON-biodegrading microorganisms without requiring the analysis of the metabolites produced. This method has a good recovery (80.2%), reproducibility (3.2%) and low limit of quantification (0.60 μg/ml) that allows quantification under a wide range of DON concentrations in microbiological culture assays.

1. Introduction

Mycotoxin contamination of food and feed is a worldwide problem for which there is still no definitive solution, despite the maximum limits that different food regulatory agencies have established. In wheat and barley, the most commonly found mycotoxin is deoxynivalenol (DON) (Wegulo, Baenziger, Hernandez Nopsa, Bockus, & Hallen-Adams, 2015), generating health problems mainly due to chronic exposure (Payros et al., 2016; Pestka, 2007). For this reason, intense research has been carried out considering several approaches for controlling DON levels (prevention, removal, degradation or processing) (Awad, Ghareeb, Böhm, & Zentek, 2010; Karlovsky et al., 2016), one of which that has attracted growing interest is biological detoxification of DON by using selected microorganisms, usually bacteria or bacterial consortia (Muhialdin, Saari, & Meor Hussin, 2020; Yao & Long, 2020; Zhu, Hassan, Lepp, Shao, & Zhou, 2017).

The mechanisms by which microorganisms can detoxify toxins have been studied for different mycotoxins. Thus, interactions with cell walls based on adsorption phenomena and enzymatic transformation are the main mechanisms described in the literature for most mycotoxins (Piotrowska, 2021). In particular, DON biotransformation products have been used to evaluate the transformation percentage of DON (Piotrowska, 2021; Wang et al., 2019; Yu et al., 2010). In addition, Jia, Cao, Liu, and Shen (2021) recently observed similar low DON contents in incubations with bacterial culture and with cell-free culture supernatant, concluding that some extracellular proteins can also play a role in DON detoxification. However, the capacity of bacterial cell walls to bind DON is also well documented (Hassan & Bullerman, 2013; Niderkorn, Morgavi, Pujos-Guillot, Tissandier, & Boudra, 2007). It is clear that in the incubations with microorganisms, DON biotransformation (if it occurs) will appear concomitant with the adsorption phenomena on cell walls. The importance of distinguishing both effects is related to the possible reversibility of the process, which is more probable in the case of binding to cell walls.

In assays aimed at identifying mycotoxin-biodegrading bacteria, a large number of samples are usually tested, and low success rates are achieved. Different strategies have been followed to identify these bacteria. Some authors have chosen to screen isolated microorganisms

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https://doi.org/10.1016/j.lwt.2022.113684
Received 11 March 2022; Received in revised form 30 May 2022; Accepted 20 June 2022
Available online 23 June 2022

by incubation in culture media in the presence of the mycotoxin (Franco, Garcia, Hirooka, Ono, & dos Santos, 2011; Niderkorn, Boudra, & Morgavi, 2006). Niderkorn et al. (2007) screened 202 bacteria and did not observe DON degradation products, with percentages of binding due to adsorption between 15 and 22%. Yu et al. (2010) used a PCR-DGGE-guided microbial selection process to identify DON-transforming bacteria from chicken intestines. They isolated 196 bacteria, and only 10 of them were found to be capable of transforming DON to DOM-1. Sato et al. (2012) used long time-stressing subculturing processes in minimal media containing the mycotoxin to induce the metabolism of the mycotoxin. From 169 environmental samples, they could only find 13 DON-biodegrading bacteria. Shima et al. (1997) only found a DON-biodegrading bacteria following a similar stress subculturing process; specifically, subcultured samples were subjected to a 2- to 8-day incubation in a medium supplemented with 200 mg of DON/ml up to 14 times. Given the low success rates found, it is necessary to test a large number of isolates to identify a DON-biodegrading strain; thus, having a simple and rapid method is especially convenient.

In general, methods aimed at identifying mycotoxin-biodegrading bacteria have been carried out using high levels of mycotoxin and bacterial inoculum (10–200 μg DON/ml, 10^8–10^{10} CFU/ml), and the degradation capacity is estimated by the analysis/detection of the remaining mycotoxin and/or the degradation products (Franco et al., 2011; Niderkorn et al., 2007, 2006 and; Sato et al., 2012; Shima et al., 1997). However, in some studies, the possible effect of adsorption of the analytes to the cell walls is not considered, so it is possible that the observed toxin reductions are a result of both biodegradation and adsorption (C. He, Fan, Liu, & Zhang, 2008; Wang et al., 2019; Wilson et al., 2017).

A few studies have included supplementary assays to evaluate the adsorption effect through incubation with inactive cells (Franco et al., 2011) or have opted to try and avoid the adsorption phenomenon instead with a second incubation in a lysis buffer (Völkl, Vogler, Schollenberger, & Karlovsky, 2004). Nevertheless, to perform screening assays with a large number of microorganisms, it is necessary to develop a simple, inexpensive, and rapid method with adequate reproducibility and recovery that allows the quantification of the total amount of analyte/s, including the dissolved and adsorbed fractions.

Therefore, the aim of this work was to establish a fast, simple and reliable method that allows the quantification of the total DON content, even the DON fraction adsorbed on the cell wall of the microorganisms, for its use in screening assays designed for searching DON-biodegrading microorganisms. With this methodology, the DON-biotransformation capacity of a bacteria (or bacteria consortium) can be evaluated by the analysis of DON remaining in the cultures, without the need to evaluate and analyse the metabolites produced.

2. Materials and methods

2.1. Chemicals

DON was provided by Romer Labs (Tulln, Austria). Methanol, acetonitrile, ethyl acetate and NaCl were purchased from Fisher Scientific (Loughborough, UK). K_2HPO_4, KH_2PO_4, $FeSO_4{\cdot}7\ H_2O$ and $CaCl_2{\cdot}2\ H_2O$ were obtained from Scharlau (Sentmenat, Spain). $MgSO_4{\cdot}7\ H_2O$ and NH_4NO_3 were obtained from Quality Chemicals (Esparreguerra, Spain). Bouillon MRS and Gelose MRS were purchased from Biokar (Beauvais, France), and L-cysteine was purchased from Aldrich (St. Louis, USA).

2.2. Bacterial cultures

To minimize the reagents needed, microbial cultures were prepared in sterile 96-well ELISA microplates. Two hundred microlitres of culture media and 50 μl of inoculum (prepared in Man, Rogosa & Sharpe medium supplemented with L-cysteine (2.5 mg/ml), named MRSc) were added to each well of the microplate. Mineral salt medium (MM) according to Sekar, Mahadevan, Sundar, and Mandal (2011) was employed as culture media with slight modifications: 1.73 g K_2HPO_4, 0.68 g K_2HPO_4, 0.1 g $MgSO_4{\cdot}7\ H_2O$, 4 g NaCl, 0.03 g $FeSO_4{\cdot}7\ H_2O$, 1 g NH_4NO_3 and 0.02 g $CaCl_2{\cdot}2\ H_2O$ in 1000 ml of water (pH adjusted to 7.0). A concentrated DON solution was also added to the MM such that the final DON concentration in each well was 30 μg/ml, unless otherwise indicated. DON was the only carbon source in the media, except for some residual MRSc constituents of the inoculum.

The microplate was incubated (37 ± 1 °C, 1 week) under anaerobic conditions achieved by employing an oxygen scavenger kit (BD GasPak EZ Anaerobe Container System; Becton, Dickinson and Company; Sparks, USA). To minimize evaporation, the microplate was sealed with parafilm, and a receptacle of water was left inside the anaerobic jar.

2.3. DON extraction

A diagram for the DON extraction methodology is shown in Fig. 1. After incubation, the entire content of each well was recovered and transferred to an Eppendorf tube containing 30 mg of NaCl.

Three consecutive extractions with 400 μl of organic solvent were carried out, withdrawing 300, 400 and 400 μl of extracting solvent for the first, second and third extractions, respectively. For each extraction cycle, the Eppendorf tube was vortexed for 30 s, ultrasound-treated for 1 min (to release the DON adsorbed on the cell walls), and vortexed for 30 s again. Therefore, a total of 1100 μl of extraction solvent was collected. Ultrasound was applied using the Bransonic M2800H-E (Branson Ultrasonic SA, Carouge, Switzerland) at maximum power.

The collected extraction solvent was evaporated at 40 °C under a gentle stream of N_2. The residue was resuspended in 0.8 ml of methanol: water 10/90 (v:v) (3.2 dilution factor), vortexed, filtered through 0.22-μm PTFE filters and analysed by HPLC-DAD.

2.4. Selection of the extraction solvent

Three different extraction solvents were tested: ethyl acetate, a 50/50 mixture of ethyl acetate and acetonitrile, and acetonitrile. The performance of the extraction using ethyl acetate but not using salt was also evaluated. Pure MRSc was employed instead of a microbial inoculum prepared in MRSc. Assays were performed in duplicate.

2.5. DON analysis

HPLC-DAD determination of DON was performed using an Agilent Technologies 1260 Infinity HPLC system (California, USA) coupled with an Agilent 1260 Infinity II Diode Array Detector (DAD). A Phenomenex® Gemini C18 column (California, USA) was used (150 × 4.6 mm, 5-μm particle size, 110 Å pore size). Three mobile phases were prepared: phase A (methanol:water 10:90, v:v), phase B (acetonitrile:water 20:80, v:v) and phase C (100% methanol). The gradient applied was as follows: 0 min 100% A; 10 min 60% A and 40% B; 13 min 60% A and 40% B; 15 min 100% C; 25 min 100% C; 29 min 100% A and 40 min 100% A (11 min were needed to clean and re-equilibrate the column). The flow rate was 1 ml/min, the column temperature was 40 °C, and the injection volume was 50 μl. The DON retention time was 10.0 min (absorbance reading at 220 nm).

2.6. Testing the method: evaluation of total DON determination

To demonstrate that the proposed method allows the extraction and analysis of the total DON (dissolved and adsorbed on cell walls) from microbial cultures, the following experiment was designed. With the hypothesis that the application of ultrasound during the extraction process releases the DON adsorbed on the cell walls, several samples were prepared and analysed following the methods described above, but half of them were extracted without applying ultrasound.

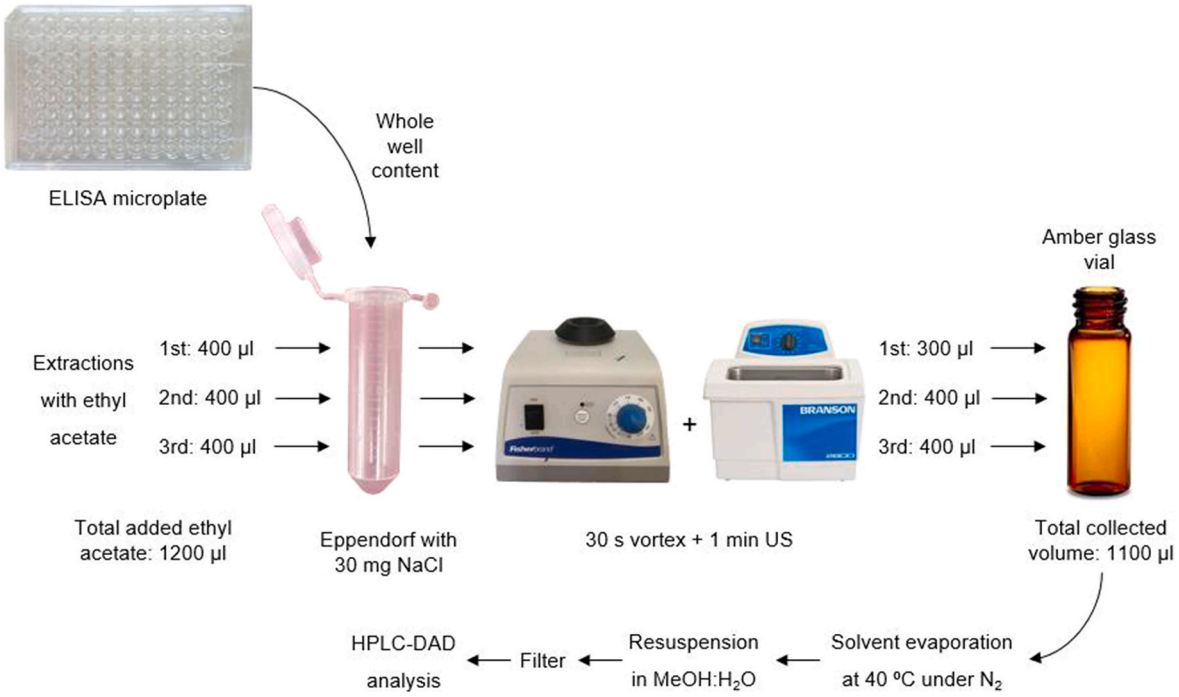

Fig. 1. DON extraction methodology diagram.

As microorganisms from the intestinal microbiota have proven to be good DON-biotransforming candidates (Gratz, Duncan, & Richardson, 2013; Guan et al., 2009; Yu et al., 2010), it was decided to use 3 intestinal colonizers: *Lactobacillus fermentum* (LF26), *Staphylococcus hominis* (SH10) and *Enterococcus faecium* (EF1), all of which were generously provided by the Probilac research group (Complutense University of Madrid, Spain).

The three microorganisms were cultivated in a microplate. Two hundred microlitres of MM and 50 µl of inoculum were mixed in each well, which also contained DON at a final concentration of 10 µg/ml (added to the MM). A control with DON but with no microorganisms was also included (50 µl of pure MRSc was added instead of 50 µl of the inoculum). Three replicates for each microorganism (and for the control) were prepared for each extraction method (with and without the application of ultrasound). The microplate was left to incubate (37 ± 1 °C, 1 week) in anaerobiosis before DON extraction and analysis.

In parallel, concentrations of the three tested microorganisms (CFU/ml) were calculated by plating dilutions of the respective inoculums in MRSc agar, which was the only method for determining the concentration of viable cells in those inoculums. Plates were incubated (37 ± 1 °C, 2 days) in anaerobiosis. Knowing the concentration of the inoculums and the proportion of the MM and inoculum in the wells, the concentration of the tested microorganisms on the well was calculated.

2.7. Validation of the method

Linearity was checked with a calibration curve of DON standards from five different concentrations (1, 5, 10, 20 and 30 µg/ml) prepared in triplicate. The standards were prepared in methanol:water 10:90 (v:v).

To determine the recovery of the method, cultures of *E. faecium* were prepared as in Section 2.2. In this case, cultures with five different DON concentrations (1, 5, 10, 20 and 30 µg/ml) were assayed in triplicate. The concentration of the inoculum (CFU/ml) was calculated by preparing a dilution series and plating them in Petri plates with MRSc agar in duplicate. Petri plates were incubated (37 ± 1 °C, 2 days) in anaerobiosis. In the case of the DON biodegradation assay, after 1 week of incubation of the microplate, toxin concentrations were determined, and the average recovery from all samples was estimated.

Reproducibility was similarly estimated. Cultures of *E. faecium* were prepared as in Section 2.2. at two different DON concentrations (5 and 20 µg/ml) on three different days, in triplicate each day. After 1 week of incubation, DON concentrations were determined, and average repeatability was calculated. The limit of detection (LOD) and quantification (LOQ) were calculated as three and ten times the signal/noise ratio using three samples incubated with *E. faecium* containing 1 µg/ml of DON.

2.8. Statistics

One-way ANOVA and least-squares difference (LSD) Fisher's tests were performed to evaluate the effects of the different sample types ($p < 0.05$). Statistical analyses were performed using STATISTICA (version 7.1) (StatSoft, Inc., 2005).

3. Results and discussion

3.1. Selection of the extraction solvent

The results of DON recovery for each extraction solvent tested are shown in Fig. 2. No significant differences between extraction solvents with added salt were observed in terms of recovery (p value = 0.54); thus, ethyl acetate was selected as the extraction solvent due to its lower boiling point and, thus, faster evaporation.

Extraction with ethyl acetate without using salt was discarded, as its recovery was lower than that achieved by adding salt (p value = 0.026). A higher recovery using salt may have been achieved due to the salting-out technique. Salt addition has previously been employed for extracting DON (Hamed, Arroyo-Manzanares, García-Campaña, & Gámiz-Gracia, 2017; Mariño-Repizo, Goicoechea, Raba, & Cerutti, 2018).

3.2. Testing the method: evaluation of total DON determination

The DON concentrations in the control and the three tested microorganisms are shown in Table 1. Half of the samples were analysed using the ultrasound-assisted extraction method (US), and the other half were analysed without applying ultrasound (N-US). The CFU/ml of

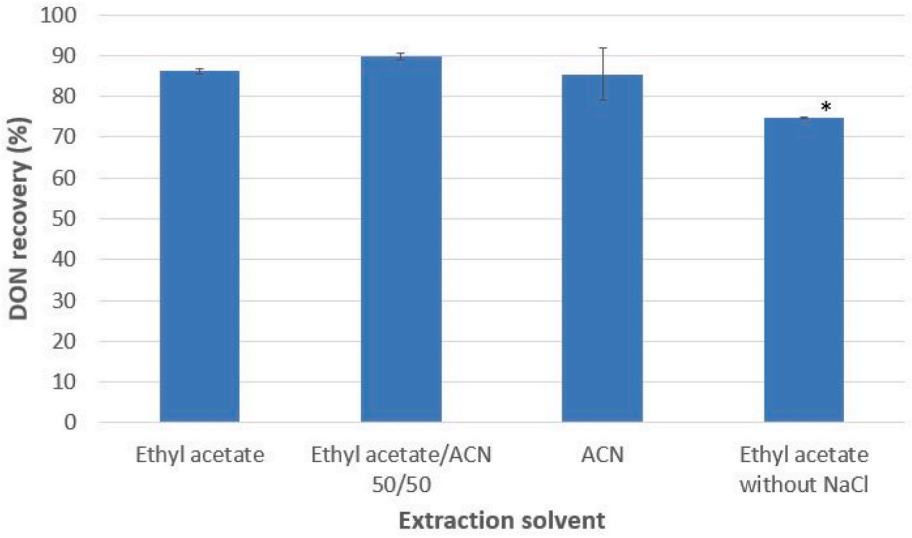

Fig. 2. DON recovery (%) for each extraction solvent tested (initial DON concentration 30 μg/ml). * Indicates statistical differences in ANOVA respect to the other samples.

Table 1
DON concentrations (μg/ml) in samples extracted with (US) and without (N-US) ultrasound-assistance, and CFU/ml of microplate microbial culture.

Sample	Extraction method		CFU/ml
	US	N-US	
Control	2.52 ± 0.06	2.30 ± 0.25	–
Lactobacillus fermentum	2.54 ± 0.06[a]	1.93 ± 0.15[b]	$1.5{\cdot}10^7$
Staphylococcus hominis	2.50 ± 0.09	2.26 ± 0.16	$3.3{\cdot}10^5$
Enterococcus faecium	2.65 ± 0.08[a]	2.38 ± 0.12[b]	$1.7{\cdot}10^7$

[a,b] Means in a row followed by different letters are significantly different at $p < 0.05$ using one-way ANOVA.

microplate microbial cultures for each microorganism are also shown.

As expected, no significant differences in DON concentrations were observed between the US and N-US control samples (p value = 0.208). Significantly lower DON concentrations were observed for the N-US samples than the US samples in the cases of *L. fermentum* and *E. faecium* (p values of 0.002 and 0.031, respectively). The lower DON concentration in the N-US samples can be explained by the adsorption of the toxin to the cell walls of the bacteria, which is a phenomenon that has been observed in numerous studies (Luo, Liu, Yuan, & Li, 2020; Yao & Long, 2020). It is also notable that standard deviations are much smaller in US-treated samples than in non-US-treated samples.

In contrast, no significant differences were observed between US and N-US extractions in the case of *S. hominis* (p value = 0.085). This can be explained by the much lower concentration of this inoculum compared with the other two (10^5 *vs.* 10^7 CFU/ml, respectively). A lower number of microorganisms most likely implies a lower cell wall surface to which DON can adsorb. Lu, Liang, and Chen (2011) demonstrated that the adsorption of zearalenone to bacteria depended on the bacterial concentration, and it is likely that the same phenomenon occurs with DON.

Other authors have used different approaches to try and determine the extent to which biodegradation phenomena are important in mycotoxin detoxification studies. For example, Kosztik, Mörtl, Székács, Kukolya, and Bata-Vidács (2020) chose to centrifuge the cultures for 40 min and analyse the supernatant on one side (20-min shaking extraction) and the cell biomass on the other side (20-min shaking extraction and 10-min centrifugation). The toxin found in the cell biomass corresponded to adsorption phenomena. If the sum of the two concentrations of mycotoxins was lower than the original concentration, it was assumed that the missing toxin had been biodegraded. Although this method works, it is more expensive and time-consuming than the method presented herein because the determination of whether one culture is capable of biodegradation requires the performance of two analyses instead of one, and long wait times are spent centrifuging and shaking.

Völkl et al. (2004) prevented the adsorption of DON to cell walls by using a lysis buffer that contained proteinase K and sodium dodecyl sulfate (SDS). Despite working, a 60-min incubation at 37 °C and centrifugation were required, again resulting in a long process regarding in comparison with the proposed method.

3.3. Validation of the method

The results for the validation of the method using ethyl acetate as the extraction solvent are shown in Table 2. For the recovery assay, it was estimated that there were 5.4×10^6 CFU/ml microplate microbial cultures, and the average recovery rate was approximately 80%. Overall, this method has proven to exhibit good recovery and reproducibility and is able to evaluate total DON levels over a wide range of concentrations. By testing microbial cultures with a DON concentration of 30 μg/ml, which is much lower than the concentrations used in many other studies (W.J. He et al., 2016; Ikunaga et al., 2011; Shima et al., 1997; Yu et al., 2010), reductions of the toxin of up to 98% can be studied (based on the obtained LOQ).

4. Conclusions

The proposed method for total DON quantification in bacterial cultures can be used for identifying bacteria capable of DON

Table 2
Validation parameters for the DON extraction method.

	DON concentration (μg/ml)					Average
	30	20	10	5	1	
Recovery (%)	80.15 ± 2.12	81.34 ± 1.39	84.24 ± 0.08	83.98 ± 7.56	71.25 ± 3.93	80.19 ± 5.94
Reproducibility[a] (%)	–	1.25	–	5.19	–	3.22
LOD[b] (μg/ml)	0.18 ± 0.01					
LOQ[b] (μg/ml)	0.60 ± 0.02					

[a] Reproducibility was calculated only for DON concentrations of 20 and 5 μg/ml, in three different days (n = 9).
[b] LOD and LOQ were calculated using a DON concentration of 1 μg/ml.

biodegradation (excluding the adsorption effect). It is fast, simple and economical, with good average recovery (80.19%) and reproducibility (3.22%), and allows the study of biodegradation phenomena over a wide range of toxin concentrations (1–30 μg/ml).

Considering that biodegradation implies a definitive chemical change in the mycotoxin structure, while adsorption of DON and other mycotoxins can be a reversible process (Adami, Tajabadi Ebrahimi, Bagheri Varzaneh, Iranbakhsh, & Akhavan Sepahi, 2020; Haskard, El-Nezami, Kankaanpää, Salminen, & Ahokas, 2001), the proposed method allows screening studies to focus only on the toxin biotransformation phenomenon and is, therefore, more adequate for use in the search for effective DON-biodegrading microorganisms.

Funding

This work was funded through the project [PID 2020-114836RB-I00] awarded by the Spanish Ministry of Science and Innovation MCIN/AEI/10.13039/501100011033. B.B.-V. and J.J.R.-B. appreciate the FD predoctoral fellowship [PRE 2018-085,278] of the Spanish Ministry of Science, Innovation and Universities, and the postdoctoral contract from AGROTECNIO-CERCA Center, respectively.

CRediT authorship contribution statement

B. Borràs-Vallverdú: Conceptualization, designed the experiment, Formal analysis, Writing – original draft. **A.J. Ramos:** Conceptualization, designed the experiment, Writing – review & editing. **S. Marín:** Writing – review & editing. **V. Sanchis:** Writing – review & editing. **J.J. Rodríguez-Bencomo:** Conceptualization, designed the experiment, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The researchers thank the Probilac research group (Complutense University of Madrid, Spain) for selflessly providing the microbial cultures.

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