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Aflatoxin B1 Binding by Lactic Acid Bacteria in Protein-Rich Plant Material Fermentation

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Featured Application: The current study provides applicable results for enhancing food safety by showing that certain starter lactic acid bacteria have the potential to mitigate background level mycotoxin risks. This is of crucial importance in the current global situation, where the mycotoxin contamination of foodstuffs is emerging due to climate change. The results of this study provide a basis for easily accessible technologies to also ensure the availability of safer and nutritious food to low-income countries.

Abstract: At the same time as the strong ambition to improve sustainability and the healthiness of food systems through a transition towards a more plant-based diet, climate change is increasing the risk of plant diseases. Consequently, mycotoxigenic fungi have become a food safety issue of major importance. A variety of strategies to suppress fungal growth in the pre- and postharvest stages of plant production have been established, and the potential of various biological methods has been assessed to ensure food safety. Of the various food microbes, lactic acid bacteria are known for their capacity to suppress the growth of toxigenic fungi and adsorb free mycotoxins. The current study showed that lactic acid fermentation could mitigate aflatoxin risk in plant-based foods through a reduction in free aflatoxin B1. In line with previous studies, in which *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) was shown to reduce the level of free aflatoxin B1 in vitro, *L. plantarum* was shown to achieve up to a 90% reduction in free aflatoxin B1 in food fermentation. The results showed that traditional lactic acid fermentation, using *L. plantarum* as the starter strain, could be applied to mitigate aflatoxin B1 contamination risk in proteinaceous plant-based foodstuffs. In a wider context, fermentation using selected strains of lactic acid bacteria as starters could also enhance the availability of nutritious and safer food in terms of mycotoxin risk in low-income countries.

Keywords: food safety; mycotoxin; aflatoxin B1; lactic acid bacteria; fermentation

Citation: Rämö, S.; Kahala, M.; Joutsjoki, V. Aflatoxin B1 Binding by Lactic Acid Bacteria in Protein-Rich Plant Material Fermentation. *Appl. Sci.* **2022**, *12*, 12769. https://doi.org/10.3390/app122412769

Academic Editor: Slawomir Ciesielski

Received: 11 November 2022
Accepted: 5 December 2022
Published: 13 December 2022

1. Introduction

There has been increasing evidence shown for the negative environmental effects and human health risks associated with the typical western diet rich in animal products through an increase in diet-related, non-communicable chronic diseases [1,2]. Livestock agriculture is the world's largest user of land resources, with the pasture and arable land dedicated to the production of feed representing almost 80% of the total agricultural land [3]. One-third of global arable land is used to grow feed, while 26% of the Earth's ice-free terrestrial surface is used for grazing [3]. Given that the global population is expected to increase to nine billion by 2050 [4], more sustainable solutions must be promoted to fulfil the growing demand for food production.

There is an increasing consensus that a transition towards a more plant-based diet together with a decreased consumption of meat and other animal-based products would improve the sustainability of food systems through the decreased use of diminishing natural resources [5,6]. However, due to climate change, the increase in plant diseases associated to toxigenic fungal species and their secondary metabolites, mycotoxins, have

become a food safety issue of major importance. Therefore, in line with the new diet trends, a risk assessment of exposure to mycotoxin may need re-evaluation if lower mycotoxin levels than the allowed maximum cannot be managed in plant-based products [7]. About 300-400 mycotoxins are known today, and they cause huge economic losses and induce health risks by contaminating agricultural commodities [8]. The United Nations Food and Agriculture Organization (FAO) has estimated that 25% of global food crops are contaminated with mycotoxins (reviewed in [9]).

Of the members of the mycotoxin group, aflatoxins (AFs) present a major food and feed safety issue. AFs are produced by several fungal species belonging to the genus *Aspergillus*; the two species of major risk are *Aspergillus flavus* and *Aspergillus paraciticus* [10]. AFs are toxic and potentially carcinogenic metabolites, including the structural analogues AFB1, AFB2, AFG1 and AFG2. The International Agency for Research on Cancer (IACR) has classified AFB1 as a Group I carcinogen, indicating carcinogenicity to humans [11]. Due to the imminent health risks, EU has set maximum levels for AFs in different foodstuffs such as baby food, different nuts and almonds, dried fruits and figs, cereal products and spices [12]. There are separate maximum levels for AFB1 and for the sum of AFB1, AFB2, AFG1 and AFG2 (AF sum). The lowest level of 0.1 μg/kg has been set for AFB1 in cereal-based baby food. The maximum level for AFB1 in cereal products is 2.0 μg/kg, and that for the sum of AFs is 4.0 μg/kg. The maximum level for AFB1 in maize products is 5.0 μg/kg, and that for the sum of AFs is 10.0 μg/kg. The best instrument for the quantification of AFs and other mycotoxins in cereal-based and other foods is liquid chromatography–tandem mass spectrometry (LC-MS/MS) utilizing the multiple reaction monitoring (MRM) technique when the lower limit of detection (LOD) and limit of quantification (LOQ) is needed such as in the infant food case [13]. This technique is the most reliable method when monitoring either nationally or EU-regulated mycotoxins in challenging matrixes such as nut-based products [14].

AFs are commonly found in cereal crops such as maize, but they can contaminate a wide variety of staple foods [15,16]. Even though tropical and subtropical areas are susceptible to severe AF outbreaks, some temperate regions such as the United States Midwest are also subject to AF contamination [17]. Yet, already in 2007, a survey conducted by the European Food Safety Authority (EFSA) indicated an emerging issue of potential aflatoxin contamination in southern Europe due to climate change [18]. As a result of hot and dry seasons, *Aspergillus flavus* has become a dominant pathogen in maize in several southern European countries, which has increased the risk of the emergence of AFB1 [19]. Moreover, there is evidence that changing environmental conditions may affect the relative expression of key regulatory and structural genes correlating with AFB1 production (reviewed in [20]), which may also have an impact on the emergence of AFB1 in Europe.

Traces of mycotoxins, remaining under the cut-off limits, often occur in crops vulnerable to fungal infestation. Thus, to mitigate prolonged background-level mycotoxin exposure, specific attention must be paid to mitigation strategies, including potential biological methods, for ensuring food safety. The use of different types of mycotoxin binders such as zeolite and silicate is typical in feeds [21], and they have also been studied in food. Li et al. found synthetic rice-husk-based MCM−41 silica to be efficient for the removal of AFB1 from peanut oil [22]. Wang et al. studied the antioxidative properties of curcumin to prevent liver damage in mice [23]. Fermentation has been used as a method of food preservation for centuries, and a huge variety of traditional fermented products can be found globally. Lactic acid bacteria (LAB) are an important group of microbes applied in the production of plant-based fermented foods in a safe and effective manner. The metabolic activity of food-fermenting LAB plays an important role in the nutritional value, sensory properties, shelf life and safety of products [24–26]. Most LABs have GRAS status (generally recognized as safe), and products made by LAB fermentation are widely accepted by consumers as natural and functional foods. In this study, *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), *Levilactobacillus brevis* (formerly *Lactobacillus brevis*) and *Pediococcus*

pentosaceus were utilized as starters in fermentation. These species have had a long history as starter cultures in milk, meat and vegetables and are frequently found in fermented plant-based products, such as sauerkraut, sourdough, pickles and brined olives [27–29]. Traditionally, a variety of legumes have been used as raw materials in numerous fermented products [30]. Fava bean (*Vicia faba* L.) belongs to the legumes family and has had a long history of use as both food and feed. It has been recognized as a promising alternative plant-based source of protein, dietary fiber, vitamins and phytochemicals [31], which has also increased its commercial importance.

Because of their capacity to suppress the growth of toxigenic fungi and adsorb free mycotoxins, several LAB strains have been used as antifungal and anti-mycotoxigenic agents (reviewed in [32,33]). In this study, the capacity of the selected strains of lactic acid bacteria to bind AFB1 in fava bean fermentation was investigated.

2. Materials and Methods

2.1. AFB1 Binding by Lactic Acid Bacteria in Fava Bean Suspension

Fava bean suspension (FBS) was prepared by mixing fava bean flour (Vihreä Härkä, Littoinen, Finland) with tap water to a concentration of 7% w/v. The suspension was pasteurized by heating at + 90 °C for 45 min and divided into 50 mL aliquots in 50 mL Falcon tubes (Thermo Fisher Scientific, MA, USA). Of the 50 mL FBS samples, twelve were spiked with 10 µg/kg AFB1. For the AFB1 binding experiment, LAB strains *L. brevis* GRL1 [34], *L. plantarum* B2 27 [35], *L. plantarum* MLBPL1 [36] and *P. pentosaceus* MF3b, originating from the German Collection of Microorganisms (ATCC 8287), isolated from fermented milk and sauerkraut and from a barley-malting process of a Finnish malthouse, respectively, were cultivated in MRS broth (Becton Dickinson, NJ, USA) at + 32 °C overnight without agitation to the late logarithmic or early stationary phase. Cultures of each strain were used to inoculate (1.5% inoculum) FBS spiked with 10 µg/kg AFB1 in Falcon tubes. For the reference sample, FBS without AFB1 addition was inoculated with *L. plantarum* B2 27. All inoculations were made in triplicate. FBS spiked with 10 µg/kg AFB1 was used as a positive control and FBS without AFB1 addition as a negative control in the experiment. All the Falcon tubes were incubated at 32 °C overnight and then placed into wet ice. A 0.1 mL sample was taken from each tube and decimal dilutions were made until a concentration of 10^{-7} was reached. For the determination of colony forming units, each dilution was plated on MRS using an easySpiral Pro® plater (Interscience, Mourjou, France). Colonies were counted after 3 days of incubation at 30 °C using a Scan 4000 colony counter (Interscience, Mourjou, France). After sampling for colony count determination, the Falcon tubes were centrifuged at 4500× g for 15 min at + 4 °C (Eppendorf Centrifuge 5804R, Hamburg, Germany) and the supernatants were collected for AFB1 analysis.

2.2. Chemicals and Reagents for AFB1 Analysis

All solvents, reagents and water used in the study were LC-MS grade. AFB1 standard was purchased from Sigma-Aldrich (St. Louis, MI, USA). Stock solution of AFB1 (100 µg/mL) was prepared in acetonitrile. Working solution (10 µg/mL) was diluted from the stock solution in acetonitrile. The working solution was used for preparing the following standard dilutions: 1 µg/mL in water for the AFB1 binding test and 1 µg/mL in acetonitrile for the calibration, which was further diluted to 0.1 µg/mL and 0.01 µg/mL in acetonitrile. These three dilutions were used for the matrix-matched and solvent calibrations for the quantification of AFB1 with ultra-high performance liquid chromatography tandem mass spectrometry (Waters Acquity UPLC-XEVO TQ MS, Milford, MA, USA).

2.3. UHPLC-MS/MS Calibration for AFB1 Analysis

Two different types of calibration samples were prepared for the UHPLC-MS/MS: (A) A multi-point matrix-matched calibration in the supernatant of the fava bean suspension without AFB1 and LAB and (B) a multi-point calibration in water. All calibration samples were prepared as real samples. Both calibration curves contained seven different

concentration levels including a zero level without AFB1. The linear quantitative area with 2 µL injection was 0.2 ng/mL–50 ng/mL. The matrix-matched curve was used for the quantification of the samples. The LOD of the method was 0.1 µg/kg and the LOQ was 0.2 µg/kg.

2.4. AFB1 Extraction from Fava Bean Supernatant and Analysis by UHPLC-MS/MS

AFB1 was extracted and analysed according to Rämö et al. [37] with minor modifications. A sample (10 mL) of supernatant derived from the binding test experiment was transferred into a 50 mL Falcon test tube with screw cap, and caffeine (125 ng) was added as an internal standard. The sample was extracted with 10 mL of acetonitrile and 200 µL of strong acetic acid by a Vortex blender for 2 min. After that, QuEChERS extraction salt (BondElut 5988−0650, Agilent Technologies, Santa Clara, CA, USA) was added. The tube was shaken vigorously until the pressure dissipated. Then, it was mixed with a Vortex blender for two minutes and finally centrifuged at 900× *g* for 5 min (Hermle Z 513, HermleLaborTechnik, Wehingen, Germany). The upper ACN phase was transferred into a glass test tube, and a 1 mL aliquot was filtered through a 0.2 µm pore size GHP Acrodisc 13 Teflon filter (Pall Corporation, Ann Arbor, MI, USA) into a sample bottle for the UHPLC-MS/MS run. Both caffeine and AFB1 were identified and quantified according to their retention times (RT) and multiple reaction monitoring (MRM) values, as published in the supplementary material (S1) of [38].

2.5. Validation of the UHPLC-MS/MS Method for AFB1 Analysis

The selectivity of the AFB1 analysis method was studied by running (a) pure extraction solvent without the internal standard, caffeine and (b) FBS extract, without AFB1 but with caffeine. No caffeine signal was detected in the acetonitrile. No AFB1 signal was detected in the acetonitrile and FBS extract. The specificity of the method was composed of the characteristic multiple reaction monitoring (MRM) values and correct retention times for both caffeine and AFB1 [32]. The correlation coefficients (r^2) of both the solvent and matrix-matched calibration curves were at least 0.99, which indicated good linearity. The matrix effect of the UHPLC-MS/MS method was studied by comparing the slopes of matrix-matched calibration curve to the solvent curve and was calculated as the ratio of signal suppression and enhancement: SSE% = 100 × (slope of matrix-matched curve/slope of solvent curve). If SSE% = 100%, no matrix effect exists; if SSE% > 100%, it means an enhanced matrix effect, and if SSE% < 100% it means a suppressed matrix effect. A minor enhanced matrix effect was detected during the first binding test UHPLC-MS/MS run (SSE% = 106%), but during the second test the enhancing effect was significant (SSE% = 157%). Because the matrix-matched calibration was used for quantification, the repeatability in and the reproducibility between tests 1 and 2 was excellent (Figure 1). There was no background MS signal of AFB1 (RT 6.7 ± 0.2 min) in the lowest calibration level (0 ng/mL AFB1 in FBS) or in the AFB1-free FBS samples, which were normally used for the calculation of the LOD (which equals the average + 3 x standard deviation) and the LOQ (which equals the average + 6 × standard deviation). So, the lowest quantitative calibration level (0.2 ng/mL AFB1 in FBS) was used as the LOQ (=0.2 µg/kg), and the following findings were made: (1) the RT was correct, (2) both quantitative (241.2) and qualifier (285.0) ions existed and (3) their ratio (1.0 ± 0.2) was correct. (4) In addition, the average signal to noise (S/N) value of AFB1 in this level was 44 (varying between 34 to 54), which was adequate for quantification. The concentrations of AFB1 in all the spiked FBS + LAB samples were higher than the LOQ, and lower concentrations of AFB1 were not detected in any of the AFB1-free samples. The calculated LOD (=LOQ/2) was 0.1 ng/mL.

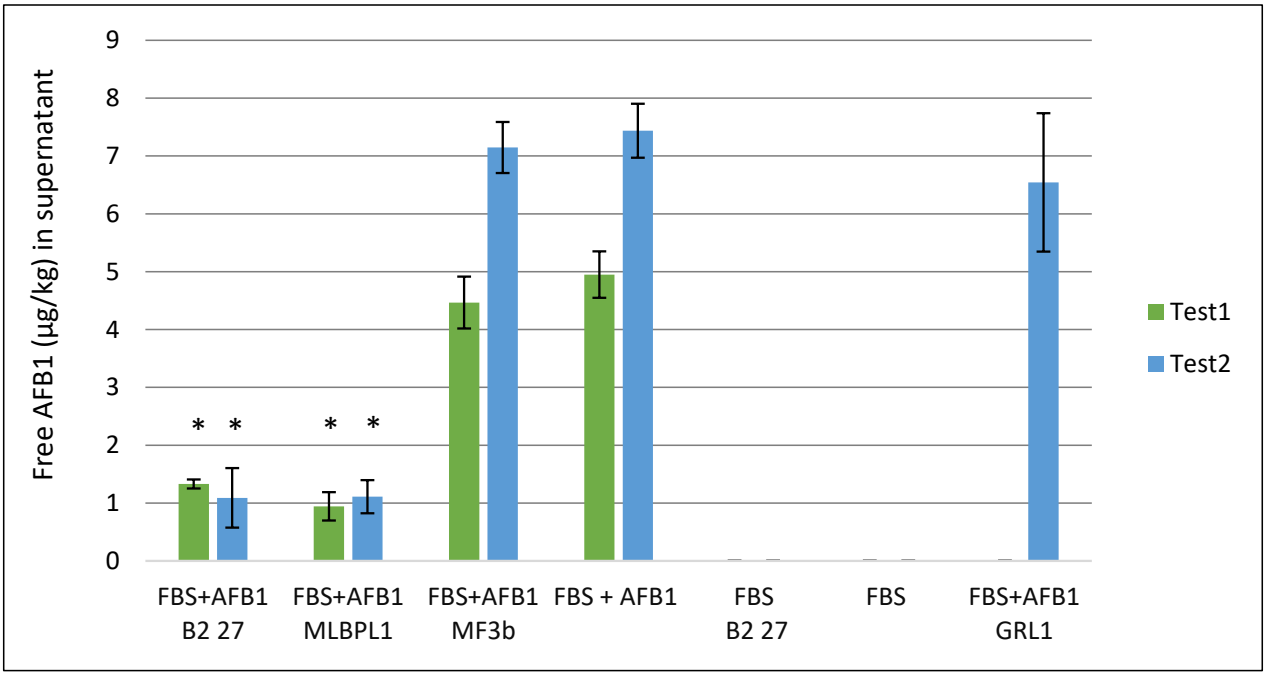

Figure 1. Concentration of free AFB1 (µg/kg) in supernatant fractions of AFB1 binding experiments. The columns show an average of three parallel cultivations, and vertical bars represent standard deviation of the mean. With the exception of *L. brevis* GRL1, the experiments were performed as two independent tests. Statistically significant differences ($p < 0.05$) with respect to background AFB1 binding without LAB inoculation (FBS + AFB1) are denoted with an asterisk *.

2.6. Statistical Analysis

Data from all experiments were expressed as the mean ± standard deviation (SD). At least three replicates were performed for each experiment. A paired-samples *t*-test was conducted to compare the differences before and after treatment in the same group [39]. The difference between the results was considered significant for *p*-values below 0.05.

3. Results

The cultivation of the LAB strains *P. pentosaceus* MF3b, *L. brevis* GRL1 and *L. plantarum* strains B2 27 and MLBPL1 in FBS spiked with 10 µg/kg AFB1 displayed no statistically significant differences ($p > 0.05$) between growth for any of the studied strains (Figure 2). The same was true with *L. plantarum* B2 27 cultivated as the growth control in FBS without AFB1 supplementation; no statistically significant difference in growth could be observed in comparison with the LAB cultures in FBS supplemented with AFB1. Microbes were encountered both in the FBS and FBS spiked with 10 µg/kg AFB1, indicating that pasteurization by heating at + 90 °C for 45 min did not fully inactivate the resilient background growth such as sporulating microbes in the FBS used as the cultivation medium for the studied LAB strains.

On the basis of the AFB1 binding experiments, *P. pentosaceus* MF3b and *L. brevis* GRL1 did not show statistically significant differences ($p > 0.05$) in AFB1 binding compared with the uninoculated FBS spiked with 10 µg/kg AFB1 (Figure 1). On the contrary, both the *L. plantarum* strains B2 27 and MLBPL1 exhibited a statistically significant ($p < 0.05$) binding of aflatoxin in the FBS spiked with 10 µg/kg AFB1. After the binding reaction, the residual free AFB1 in the suspension was about 10% of the initial added AFB1 concentration, indicating a binding efficiency of about 90%.

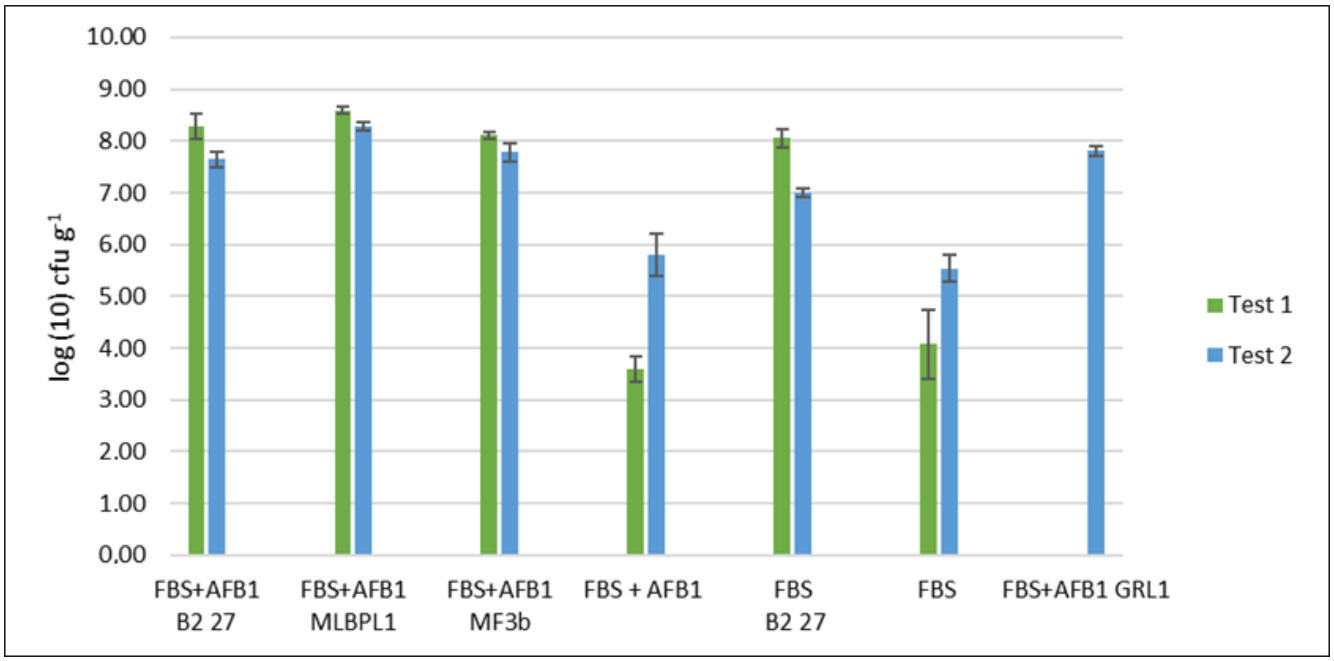

Figure 2. Growth of lactic acid bacteria (LAB) in fava bean suspension (FBS) and FBS spiked with 10 mg/kg aflatoxin B1 (AFB1). The columns show an average of three parallel cultivations, and vertical bars represent standard deviation of the mean. With the exception of *L. brevis* GRL1, the experiments were performed as two independent tests.

4. Discussion

The mechanism of LAB binding mycotoxins is not fully elucidated, and the most prevalent theory is that it is due to physical adsorption between the cell wall of the microorganism and the mycotoxin molecule. The mechanism of adsorption is assumed to be non-covalent, i.e., based on van der Waals, hydrophobic and hydrogen bond interactions [40]. A number of AFB1 binding experiments have been carried out using probiotic LAB, and the results seem to be strain-specific, with the binding efficacy ranging from 0.9 to 100% [41]. Of the LAB species frequently found in fermented plant-based products, *L. plantarum* showed a statistically significant AFB1 binding capacity in this study. All the strains tested grew to a similar cell density in the 7% fava bean suspension, indicating that the differences in the free AFB1 level determined after cultivation were not a result of the varying cell density during the cultivation procedure. A possible reason for the statistically insignificant AFB1 binding capacity of *P. pentosaceus* could be due to exopolysaccharides or its smaller cell size, which provides less free surface area on its cell wall compared to lactobacilli. As for the tested lactobacillar species, there was a notable difference between *L. plantarum* and *L. brevis*. On the basis of the cell wall structure, the difference could result from the availability of free surface area on the cell wall. *L. brevis* has been shown to possess an S-layer, a proteinaceous cell envelope structure. The proteinaceous subunits assemble on the surface of the cell forming a lattice covering the cell wall, which is mainly composed of peptidoglycan [42]. Thus, the S-layer of *L. brevis* may create a physical barrier, thus hindering the non-covalent adsorption of AFB1 to the cell wall.

In previous in vitro studies, heat-inactivated *L. plantarum* cells have been reported to reduce the level of free AFB1 by 50% during a 5–15 min incubation time [43], and an AFB1 binding rate ranging from 20.88% to 59.44% was achieved with viable and heat-inactivated cells after a 30 min incubation [44]. A longer incubation period of 24 h with viable *L. plantarum* cells has been reported to increase the AFB1 in vitro removal ratio to 89.5% [45]. In the present study, a similar AFB1 removal ratio was achieved by fermenting a fava bean suspension, which mimics a genuine food processing or preservation process. Similar results were obtained with both *L. plantarum* strains tested; no strain-specific differences

in the capacity of removing free AFB1 could be detected, even though the studied strains originated from very different ecological niches.

Besides being a traditional means to increase the shelf life, nutritional value and sensory properties of food, lactic acid fermentation has gained popularity as a minimal and ecological processing method corresponding with the emerging diet trend favoring sustainability and healthiness. In addition to this, the results of this study suggested that the traditional lactic acid fermentation of proteinaceous plant material by selected LAB starter strains may contribute to the mitigation of risk for background-level AFB1 exposure. Even though fermentation is not capable of replacing the need for antifungal production procedures or mycotoxin controls as the primary means to control mycotoxin risks, it contributes to ensuring health and well-being by mitigating prolonged background-level mycotoxin exposure. In specific agricultural practices such as organic plant production, mycotoxins may pose a notable problem due to the forbidden use of antifungal agrochemicals. Thus, specific attention must be paid to mitigation strategies, and biological methods allowed in organic production should also be harnessed for ensuring food safety at appropriate stages.

According to the Intergovernmental Panel on Climate Change (IPCC), the African continent will be the most affected by climate change in terms of temperature and weather conditions [46]. Prevailing climate conditions have been shown to increase aflatoxin risk in tropical and sub-tropical regions, which urges the development and implementation of aflatoxin control measures encompassing all value chains of food- and feed-based commodities prone to aflatoxin contamination. Good agricultural practices (GAP) have proven to be an effective technology for mitigating and managing aflatoxin risk under farm conditions. However, potential biocontrol methods will also be needed to supplement the choice of methods for the decontamination of aflatoxin (reviewed in [47]). Based on the ability of non-aflatoxigenic fungal strains to reduce aflatoxin contamination in cotton seeds, peanuts and maize, biocontrol agents for commercial applications have been developed [48]. Other potential biocontrol methods for the decontamination of aflatoxin include certain micro-organisms being used as binders, which can be added to animal feeds or even human foods. Microbial starters have been successfully used for fodder conservation and mycotoxin control in silage making [49], and studies carried out in Uganda, Tanzania and Ethiopia have suggested that using selected *Lactobacillus* strains as starters for fermentation reduced aflatoxin levels efficiently in milk and traditional maize-based fermented foods [50–52].

Overall, the potential of LAB to mitigate background level mycotoxin risks in the current global situation, where contamination of foodstuffs by mycotoxin is emerging due to climate change, further improves the applicability of fermentation as a safe and healthy food processing and preservation method. From a future perspective, the results of the current study could provide a basis for an established and easily accessible technology, which could also ensure the availability of nutritious and safer food in terms of mycotoxin risk in low-income countries.

5. Conclusions

In this study, we showed for the first time that the fermentation of a proteinaceous plant-based foodstuff with *L. plantarum* as stater culture significantly reduced the level of free aflatoxin B1. The reduction level of free aflatoxin B1 achieved in the fermentation was up to 90%. In earlier in vitro studies, the results of a similar type were obtained when live or heat-inactivated *L. plantarum* cells were incubated with free aflatoxin B1 for various time periods. In the current study, a high aflatoxin B1 removal rate was achieved by fermenting a fava bean suspension, which mimicked a genuine food processing or preservation process.

In the current global situation of climate change and emerging mycotoxin risks, biological control methods may be a valuable asset for mitigating prolonged background-level mycotoxin exposure. This is true especially in specific agricultural practices such as organic farming, which do not allow the use of chemical antifungal agents. In the global context, fermentation using specific LAB strains as starters could provide a basis for an established

and easily accessible technology to also enhance the availability of nutritious and safer food in terms of mycotoxin risk in low-income countries.

Author Contributions: Conceptualization, S.R., M.K. and V.J.; methodology, S.R., M.K. and V.J.; validation, S.R.; formal analysis, S.R., M.K. and V.J.; investigation, S.R., M.K. and V.J.; resources, S.R., M.K. and V.J.; data curation, S.R., M.K. and V.J.; writing—original draft preparation, S.R., M.K. and V.J.; writing—review and editing, S.R., M.K. and V.J.; visualization, S.R., M.K. and V.J.; supervision, V.J.; project administration, M.K.; funding acquisition, M.K. and V.J. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by strategic research funding from the Natural Resources Institute Finland.

Data Availability Statement: Natural Resources Institute Finland (Luke) is self-archiving reports and articles in an open repository, Jukuri (https://jukuri.luke.fi/, accessed on 4 December 2022).

Acknowledgments: The authors wish to thank Sari Lassila and Leena Holkeri for their skillful technical assistance in microbiological and mycotoxin analyses.

Conflicts of Interest: The authors declare that they have no conflict of interests.

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