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Research Article

Potential antifungal activity of acetic acid bacteria isolated from Vietnamese fermented cocoa beans

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Abstract

Aspergillus sp. and *Fusarium* sp. are known for the mycotoxigenic potential fungi, and are found in agricultural products, animal feed, etc. Therefore, prevention of mycotoxin hazard needs to be considered and thoroughly handled. Current food preservation methods are mainly based on chemical way, which are not environmentally friendly, persist in food and humans for a long time, and cause adverse effects on health. The biological method aligned with current trends, in which organic acid-producing bacteria are considered a potential solution because of their ability to extend the preservation time, safety level, maintain the food structure and other health benefits. In Vietnam, there was no study investigating the antifungal activity of acetic acid bacteria. Therefore, this study focused on evaluating the potentiality of antifungal activities against *Aspergillus flavus* UBOCC-A-10866 and *Fusarium foetens* M79 of 13 AAB strains isolated from Vietnamese fermented cocoa beans from Dak Lak, Ba Ria-Vung Tau and Dong Nai provinces. The results showed that acetic acid bacteria strains were all capable of inhibiting fungal growth (from 23-58% of mycelium diameter after 7 days), in which 03 AAB strains (G60T96, M61N96 and G62N24) were selected due to their great fungi inhibition activities. The minimum inhibitory concentrations of cell-free supernatant of these strains were determined from 0.32 to 0.64 mg/mL.

Keywords: *Aspergillus flavus, acetic acid bacteria, antifungal activity, Fusarium, Vietnamese fermented cocoa beans.*

1. INTRODUCTION

Fungi are widely distributed in nature and actively participate in material cycles, especially the process of decomposing organic matter and forming humus. However, fungi present as one of the main reasons causing the post-harvest losses worldwide. Several fungi as *Aspergillus*, *Alternaria*, *Fusarium*, *Cladosporium*, *Penicillium*, *Mucor*, *Rhizopus*… are found in a diverse agriculture and food products. Amongst them, *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. are common fungi species producing mycotoxin. According to the Food and Agriculture Organization of the United Nations, it is reported that about 25% of food production worldwide is contaminated with mycotoxins. These threats associated with mycotoxins in livestock have increased in most regions of the world, through the analysis of more than 14,000 samples including 3715 finished feed samples and raw materials originating from 54 countries in January to March 2017 [1]. The East Asia region has the highest risk level of 92%. Vietnam is also a country in the region with a fairly serious risk level (70%) [1]. Human and animal health can be adversely affected by the consumption of food products contaminated with mycotoxins. Furthermore, food contaminated with mycotoxins also causes heavy economic losses. Annual losses in the United States and Canada due to the impact of mycotoxins on food and livestock are estimated at $5 billion [2]. Two of the most popular mycotoxins: aflatoxin is produced by *Aspergillus* spp. especially by *A. flavus*; fumonisin is produced by *Fusarium* spp. [2].

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Beside the physical treatment, chemical preservatives and fungicides are popularly used to limite the fungi growth and food losses during preservation and extend the food shelf life. However, the chemical method may cause negative impacts on human health even on the environment, so consumers are now looking for healthier, less processed and "preservative-free" products. Therefore, biopreservation is a viable solution to increase the range of natural food products. Biopreservation is a technique to extend the shelf life of food by using natural compounds and/or safe microorganisms and/or microorganism metabolites. This preservation method is gradually becoming popular due to its ecologically benign approach [3]. Participating in this trend, research focusing on the antifungal activities of organic acid-producing bacteria, mainly lactic acid bacteria (LAB), are gradually proving their potential roles in food preservation as well as figuring out the antifungal mechanisms [4, 5]. These studies also demonstrated that antifungal activities of LAB were the combined result of several mechanisms: destabilize of fungal cell wall, change in proton gradient, produce inhibitory enzymes and induce oxidative stress [6]. The use of LAB as antifungal preservatives has been studied on a variety of food products, including bread, dairy products, fruits, and fresh vegetables [7-10]. However, there have been very few studies on the antifungal ability of acetic acid bacteria (AAB), only a few reports indicated the effectiveness of their metabolites in antibacterial activity. Research by Chu Thi Thanh Huyen et al. (2020) showed that 5% acetic acid was able to inhibit the growth of blue mold and white mold without affecting the growth of oyster mushrooms [11]. The main metabolite of AAB is acetic acid, which presents an antibacterial activity, however, studies have shown that acetic acid is not the only active compound [12]. It has been reported that acetic acid bacteria can produce various antibacterial compounds including antibacterial polysaccharides, exopolysaccharides, ascorbic acid, protocatechuic acid, and phenolic compounds [13]. The antibacterial activity of AAB is species-dependent. For example, *Acetobacter cibinongensis* and *Acetobacter syzygii* showed a significantly broader spectrum of antibacterial activity than *Acetobacter indonesiensis* [14]. The antibacterial activity of AAB is still poorly understood compared to that of LAB. Future studies should be conducted to elucidate the underlying antifungal and antibacterial mechanisms of the metabolites synthesized by AAB.

Cocoa tree (*Theobroma cacao*) is one of the most popular industrial plants in the Central Highland of Vietnam. Cocoa bean fermentation is carried out by a series of specific microorganisms including yeast, lactic acid bacteria and acetic acid bacteria. During the first 24-48 hours, yeasts are the dominant microorganisms, then, the growth of LAB increases after 24-72 hours followed by AAB growth. The fermentation process involves both anaerobic and aerobic fermentation, creating such diversity for LAB and AAB. Many AAB species have also been isolated such as *Acetobacter pasteurianus, Acetobacter tropicalis, Acetobacter senegalensis, Acetobacter lovaniensis*...[15]. The diversity of LAB and AAB contributes to the diversity of antifungal compounds, and they have a synergistic effect, thereby effectively inhibiting fungi during cocoa fermentation.

In Vietnam, research on the antifungal activities of acetic acid bacteria has not been reported in the literature. Despite the microbial diversity of Vietnamese fermented cocoa beans were investigated however, there has been no research on the application in antifungal biological control of acetic acid bacteria isolated from indigenous Vietnamese microflora. Therefor, this study aims to: (i) evaluate the antifungal activity (anti-*Aspergillus* and -*Fusarium*) of AAB strains isolated from Vietnamese fermented cocoa beans and (ii) determine the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of potential strains.

2. MATERIAL AND METHOD

2.1. Material

Thirteen AAB strains were isolated from Vietnamese fermented cocoa beans collecting from different geographic zone of Dak Lak, Ba Ria-Vung Tau and Dong Nai provinces. AAB strains were isolated in GYEC media (glucose 10 g/L, yeast extract 10 g/L, $CaCO_3$ 5 g/L, ethanol 20 mL/L, agar 2%) presenting with clear $CaCO_3$ degradation zones and Gram negative and calalase positive that were selected to evaluate the antifungal activity.

Aspergillus flavus UBOCC-A-10866 from the Collection of Université de Bretagne occidentale (Brest, France) and *Fusarium foetens* M79 provided by Food Industries Research Institute (Vietnam) were used in this study as the control sample in evaluating the antifungal activity of isolated bacterial strains (**Figure 1**). These fungal strains were chosen because of its popular infection in many grains and seeds at pre- and post-harvest, which includes cocoa beans [16].

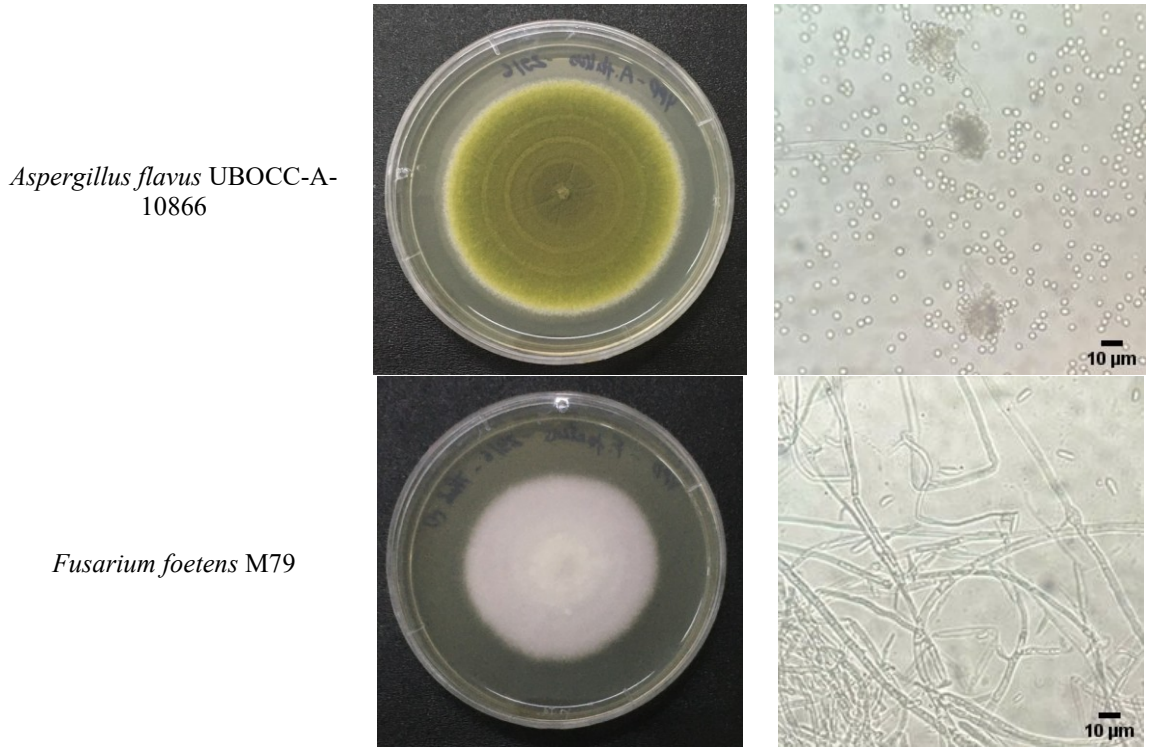

Figure 1. *Morphology of A. flavus UBOCC-A-10866 and Fusarium foetens M79 (7 days old) on YPD agar plates (Left: vegetative mycelium – Petri dish diameter of 9 cm; Right: spores under microscopy x40)*

2.2. Method

2.2.1. Microorganism cultivations

Presumptive AAB strains were inoculated in GY media (D-glucose 50 g/L, yeast extract 10 g/L and agar 20 g/L) at 30°C, 150 rpm for 24 hours. After activation, the bacterial cultures were streak-plate inoculated on GY_{Ca} ($CaCO_3$ 5 g/L) agar and then inoculated for 72 h until bacterial colonies were observable. The bacterial agar plates were then stored in the refrigerator at 2-5°C. Bacteria colonies on these agar plates were transferred onto new GY_{Ca} agar plates every week.

Aspergillus flavus UBOCC-A-10866 and *Fusarium foetens* M79 were inoculated on YPD (Himedia, India) agar plates by spot technique [17] with slight modification and incubated at 30°C for 7 days until the sporulation stage. The agar plates were then stored in the refrigerator at 2-5°C until use and transferred onto new YPD agar plates once a month.

Fungi spores were directly counted using a hemocytometer [18]. The number of spores per mL of the suspension was calculated using the Eq. 1 below

$$N\left(\frac{CFU}{mL}\right) = \frac{a \times 1000 \times D_f}{h \times S} \ (Eq.1)$$

In which:

a: the average number of spores in a square

D_f: the dilution factor of the diluted spore suspension

h: the depth of the counting grid

S: the total area of the square from which the average number of spores were counted

1000: the conversion factor between mL and mm^3

2.2.2. Evaluation of antifungal activity of AAB in agar plates

AAB strains were inoculated in GY broth and incubated at 30°C, 150 rpm for 24 hours before refrigeration centrifugation at 4000 rpm and 4°C for 10 minutes to separate the biomass and supernatant. The cell biomass

was then collected and rinsed using NaCl 0.9%. After that, the biomass suspension was adjusted to OD600 = 0.1 (equivalent to 10^6 CFU/mL) using NaCl 0.9% as blank control as well as diluent.

100 µL of the post-adjusted suspension were then inoculated on YPD agar using the spread plate method. After these agar plates were incubated at 30°C for 24 h, 7-day-old *A. flavus* and *F. foetens* spores were inoculated in the center of these plates using the spot inoculation method. The agar plates were then incubated at 30°C for 7 days. The fungi colonies were observed, and the diameter of the mycelium was measured daily, as well as the spore-forming date was recorded. YPD agar plates inoculated with only fungi were used as control samples. The experiments were carried out in triplicate. The significant difference of mean values was assessed with one-way analysis of variance (ANOVA) followed by Tukey's test using SPSS software at a significance level of $p < 0.05$.

The antifungal activity was expressed in the inhibition rate (IR) using the Eq. 2 below [19]:

$$IR\ (\%) = \frac{D_{control} - D_{sample}}{D_{KC}} \times 100\%\ (Eq.2)$$

In which: $D_{control}$ is the diameter of the mycelium on the control sample plate (mm); D_{sample} is the diameter of the mycelium on the test sample plate (mm).

2.2.3. Determination of the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of AAB's cell-free supernatant (CFS)

Preparation of CFS

AAB was activated by inoculation in GY broth for 24 h, 150 rpm, 30°C then inoculated second time in GY broth for 72 h, 150 rpm, 30°C and centrifuged at 6000 rpm, 4°C for 10 minutes to collect the supernatant. For obtening CFS, the supernatant was then filtered by 0.22 µm.

Determination of the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC)

The MIC value of CFS (cell-free supernatant contains the total metabolites) was determined by the microdilution method utilizing the 96-well microplates [20, 21]. A total volume of 250 µL solution consisting of 160 µL acetic bacteria CFS (at different dilution), 25 µL of spore suspension (10^5 spores/mL) and 65 µL of YPD broth was added to each microwell so that the final spore density of all microwells was 10^4 spores/mL. Positive control microwell contained: 65 µL of YPD broth, 160 µL sterile distilled water and 25 µL spore suspension. Negative control microwell contained: 65 µL of YPD broth, 160 µL sterile distilled water (containing cycoheximide 0.5 µg/mL) and 25 µL of spore suspension. The 96-well microplates were incubated at 30°C for 3 days for observation. The growth of fungi on all microwells was then observed to determine the microwells without visible fungal growth. The concentration of bacterial CFS in the microwell in which there was no visible fungal growth was the minimum inhibitory concentration – MIC value.

To determine the MFC value, 10 µL of the concentration corresponding to the MIC and higher concentrations evaluated were recultivated on YPD agar plates. The recultivated cultures were then incubated again at 30°C for 3 days for observation. The MFC value was determined as the concentration of CFS that avoided any visible fungal growth of the recultivated culture [22].

3. RESULTS

3.1. Evaluation of antifungal activity of AAB strains on the agar plates

The cell biomass of 13 presumptive AAB strains were tested for antifungal activity against *A. flavus* UBOCC-A-108066 (**Figure 2**) and *F. foetens* M79 (**Figure 3**) in this experiment. Considering *Aspergillus flavus*, for the control, the fungi formed fast-growing colonies of white color, which gradually turned yellowish, greenish, or brownish due to spore-forming in the later days. The spore appeared from day 2 of inoculation. For AAB samples, the mycelium diameters increased from slightly to significantly every day, depending on strains. Interestingly, the diameters of fungi mycelium in all agar plates inoculated with AAB cell biomass were visually smaller compared to that of the control. After 7 days, the mycelium diameter of the control reached 78.5 mm whereas the diameter of the AAB samples varied from 38.90 to 60.25 mm (**Table 1**). Besides, the delay of sporulation of at least one day was observed for all 13 samples. The similar trend was also observed

for anti-*Fusarium foetens* M79. After 7 days of inoculation, the control presented a full diameter of 69.67 mm, the experimental petri dishes still exhibited significantly smaller mycelium diameters ranging from 29.33 to 48.83 mm (**Table 1**). Considering the spore formation delay, all 13 strains can cause of delay of at least one day comparing to the control. The differences of antifungal activities of 13 AAB strains reflect the distinct fungal inhibition mechanisms based on the wide range of metabolite products during AAB strain growth.

Regrading the inhibition rate (IR, **Table 1**), 6 of 13 AAB strains were categorized as exerting a significant *Aspergillus*-inhibition activities and 5 of 13 AAB exhibited a significant *Fusarium*-inhibition activities (reducing the diameters of fungi higher than 35%). In the combination, three AAB strains including G60T96, M61N96, G62N24 presented the best antifungal activity for both *Aspergillus* and *Fusarium* species. These species were isolated from fermented cocoa beans in Chau Duc district (Ba Ria-Vung Tau province) and Dinh Quan district (Dong Nai province), which may propose a hypothesis that geographical origin does not necessarily have a connection with antifungal activity of AAB species, particularly in the context of this study.

The antifungal activity of AAB strain was rarely reported in the literature. Fortunately, our results are aligned with the research of Rahayu et. al., (2021) utilizing indigenous species fermented cocoa beans, namely *Candida famata* HY-37, *L. plantarum* HL-15, and *Acetobacter* spp. HA-37, as starting culture could inhibit ochratoxin-A-producing fungi [23]. However, no published studies have proven the direct inhibitory effect of AAB species on fungi growth. Therefore, these screening results could strengthen the hypothetical assumption of antifungal activity by AAB species. In the broader comparison, there were also very limited previous studies on the anti-*Aspergillus flavus* and *Fusarium* activity of LAB species evaluating the inhibition rate based on mycelium diameter. However, the results for other fungi demonstrated the similar trend with our observation. Mpeluza A et al. (2023) studied the antifungal activity of LAB species against *Colletotrichum gloeosporioides* and found that several strains can inhibit mold growth from 80.34% up to 100% [24]. Besides, Riolo M et al. (2023) concluded that *A. flavus* was more difficult to be inhibited by LAB species comparing to *C. gloeosporioides* species (RD9B and C2) [25].

Therefore, all of 13 AAB strains demonstrated a potentiality of antifungal activity. Amongst them three strains: G60T96, M61N96, G62N24 were selected for cell-free supernatant (CFS) analysis in the next experiment.

***Table 1**. Mycelium diameter and fungal inhibition rate of different AAB strains dishes after 7 days of incubation*

Strain	***A. flavus* UBOCC-A-108066**		***F. foetens* M79**	
	Diameter after 7 days, mm	Inhibition rate (IR), %	Diameter after 7 days, mm	Inhibition rate (IR), %
Control	$78.50^{a} \pm 0.71$	-	$69.67^{a} \pm 1.49$	-
M3G75	$50.50^{d} \pm 0.71$	35.67	$46.00^{bc} \pm 0.71$	33.97
ABS1	$51.00^{d} \pm 1.87$	35.03	$45.75^{bc} \pm 1.06$	34.33
G53E96	$59.25^{b} \pm 0.35$	24.52	$47.50^{bc} \pm 0.71$	31.82
G53E72	$54.75^{c} \pm 0.35$	30.25	$48.83^{b} \pm 1.08$	29.91
G62N24	$50.00^{d} \pm 0.71$	36.31	$41.75^{d} \pm 0.35$	40.07
M61N24	$52.00^{cd} \pm 0.71$	33.76	$40.00^{d} \pm 0.71$	42.59
Y63N24	$60.25^{b} \pm 0.35$	23.25	$47.00^{bc} \pm 0.71$	32.54
M61N96	$50.83^{d} \pm 2.27$	35.24	$39.75^{d} \pm 0.35$	42.95
M52N120	$54.25^{c} \pm 0.35$	30.89	$45.67^{c} \pm 1.08$	34.45
Y61T96	$57.75^{b} \pm 0.35$	26.43	$47.17^{bc} \pm 1.78$	32.30
M63T96	$52.25^{cd} \pm 0.35$	33.44	$40.75^{d} \pm 0.35$	41.51
M61T96	$52.15^{cd} \pm 0.71$	35.03	$46.10^{bc} \pm 1.36$	33.83
G60T96	$38.90^{e} \pm 0.35$	49.56	$29.33^{e} \pm 1.47$	57.90

Values with a different letter in the same column are significantly different ($p < 0.05$) according to Tukey's test

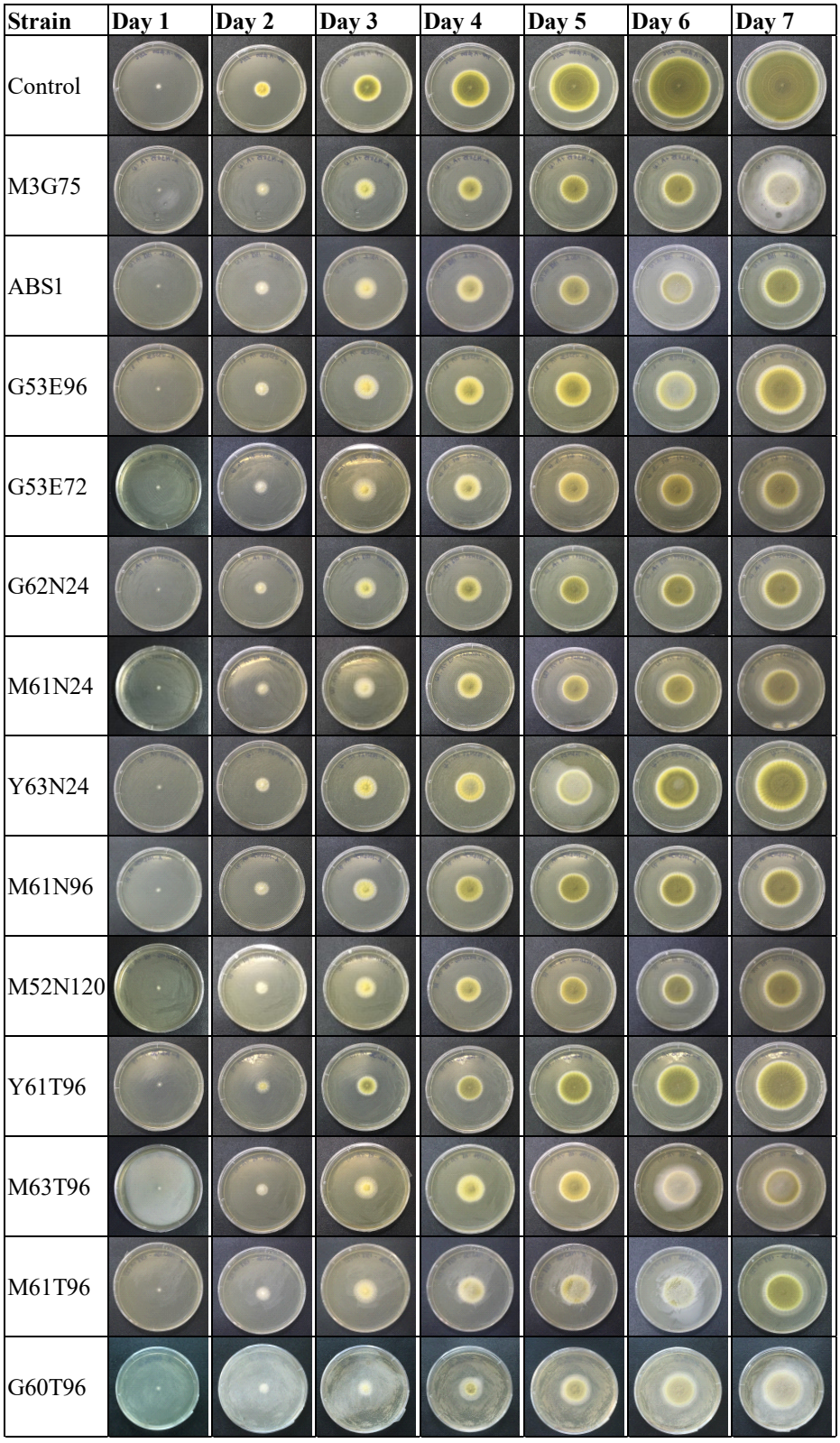

***Figure 2**. Growth of A. flavus UBOCC-A-108066 within 7 days of co-incubation with different AAB species on YPD agar plates (Petri dish diameter of 9 cm)*

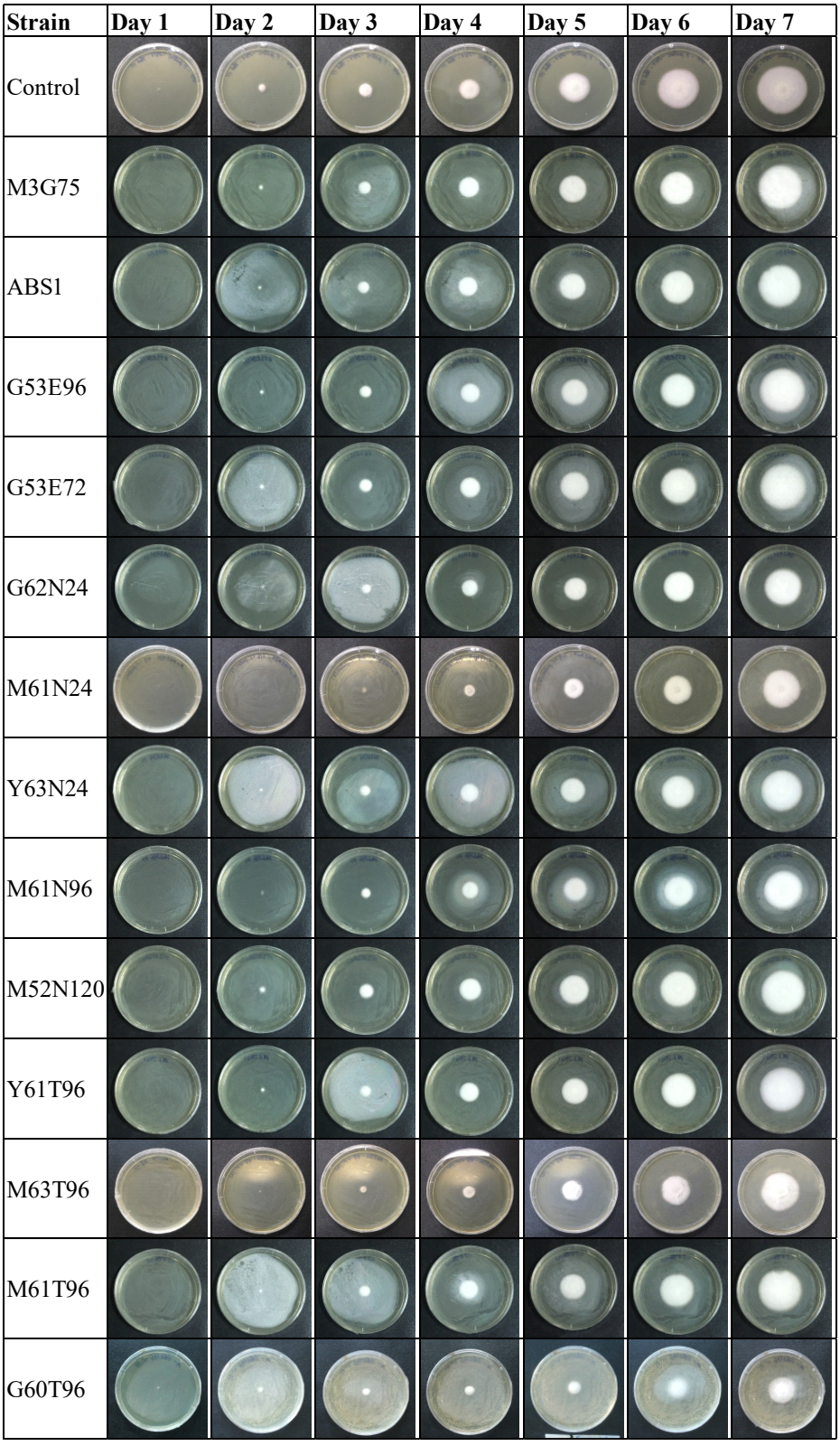

Figure 3. *Growth of F. foetens M79 within 7 days of co-incubation with different AAB species on YPD agar plates (Petri dish diameter of 9 cm)*

3.2. The minimum inhibitory concentration and the minimum fungicidal concentration of AAB's cell-free supernatant

The growth of fungi on the 96-well plate after 03 days was followed. In the negative control, the cycloheximide inhibited fungi so no growth was observed, while *A. flavus* and *F. foetens* formed yellowish green and white mycelium on the surface of the positive control well, respectively. For the samples, with *A. flavus,* only the first wells appeared no fungi growth that proves the minimum inhibitory concentration for *A. flavus* (10^4 spores/mL) of three AAB strains were 0.64 mg/mL (**Table 2**). In contrast, for *F. foetens* the minimum inhibitory concentration for G60T96 and G62N24 strains were lower than that of M61N96 strain and that of MIC for *A. flavus*. These values reached 0.32 mg/mL. Our results of MIC highlight one more time the strain dependency for antifungal activity of acetic acid bacteria and the difficulty of inhibiting *Aspergillus flavus*.

***Table 2**. MIC and MFC of three AAB strains*

Strain	pH	*A. flavus* UBOCC-A-108066		*F. foetens* M79	
		MIC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MFC (mg/mL)
G60T96	3.28	0.64	>0.64	0.32	>0.64
M61N96	3.16	0.64	>0.64	0.64	>0.64
G62N24	2.98	0.64	>0.64	0.32	>0.64

Next, to determine the minimum fungicidal concentration (MFC), 10 µL of culture from the wells containing three AAB strains (where no fungi growth was observed) were inoculated on YPD agar and incubated at 30°C for 3 days. In this experiment, all petri dishes appeared fungi growth indicating the MFC of all AAB strains for both fungi are higher than 0.64 mg/mL. As mentioned above, there was no study investigating the MIC and MFC of AAB against fungi, so our results are the first research determining the MIC of AAB strain for fungi. In the broader comparison, our results are in the same value range with MIC of LAB (*Lactobacillus plantarum)* strains against $5x10^3$ spores/mL of *A. flavus* ITEM 8111 of 0.250 mg/mL and the MFC was 0.250 mg/mL [22]. Interestingly, three AAB strains were identified by 16S rRNA sequencing (detailed result not shown) and belong to three different genus including G60T96: *Komagataeibacter saccharivorans,* M61N96: *Acetobacter pasteurianus,* G62N24: *Gluconobacter frateurii*.

4. CONCLUSIONS

The study is the first to evaluate the antifungal activity of 13 acetic acid bacteria strains isolated from fermented Vietnamese cocoa beans. All acetic acid bacteria strains demonstrated the antifungal potential against *Aspergillus flavus* and *Fusarium foetens.* Three acetic acid bacteria strains were selected: G60T96, M61N96 and G62N24 for strong anti-*Aspergillus flavus* UBOCC-A-108066 and *Fusarium foetens* M79 activities during competitive cultivation on YPD agar. The cell-free supernatant of these strains was evaluated, thereby determining the minimum inhibitory concentrations from 0.32 to 0.64 mg/mL. Our results confirm the potentiality of antifungal activity of Vietnamese indigenous acetic acid bacteria and open the potential for its application of fungi in the preservation of agricultural products and foods.

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