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Screening of lactic acid bacteria and yeast strains to select adapted anti-fungal co-cultures for cocoa bean fermentation

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ABSTRACT

Contamination with filamentous fungi during cocoa bean fermentation and drying reduces the quality of cocoa beans and poses a health risk for consumers due to the potential accumulation of mycotoxins. The aim of this study was to develop anti-fungal lactic acid bacteria (LAB)-yeast co-cultures by selecting anti-fungal strains best adapted to the cocoa bean fermentation process from 362 LAB and 384 yeast strains isolated from cocoa bean post-harvest processes. The applied multiphasic screening approach included anti-fungal activity tests *in vitro* and *in vivo* and assessment of the carbon metabolism and stress tolerance of the anti-fungal strains in a cocoa pulp simulation medium. The anti-fungal strains, *Lactobacillus fermentum* M017, *Lb. fermentum* 223, *Hanseniaspora opuntiae* H17, and *Saccharomyces cerevisiae* H290, were selected based on their high fungal growth inhibition capacity and their well-adapted metabolism. Up to seven filamentous fungal strains of the genera *Aspergillus*, *Penicillium*, and *Gibberella* were inhibited on average by 63 and 75% of the maximal inhibition zone by M017 and 223, respectively, and by 25 and 31% by the strains H17 and H290, respectively. Both *Lb. fermentum* strains converted the medium's glucose, fructose, and citric acid into 20.4–23.0 g/l of mannitol, 3.9–6.2 g/l acetic acid, and 8.6–10.3 g/l lactic acid, whereas the two yeast strains metabolized glucose and fructose to produce 7.4–18.4 g/l of ethanol. The *Lb. fermentum* strains were further characterized as particularly tolerant towards ethanol, acetic acid, and heat stress and both yeast strains tolerated high amounts of ethanol and lactic acid in the medium. Finally, the anti-fungal *in vivo* assays revealed that the two *Lb. fermentum* strains completely inhibited growth of the citrinin-producing strain, *P. citrinum* S005, and the potentially fumonisin-producing strain, *G. moniliformis* S003, on the surface of cocoa beans. Furthermore, growth of the aflatoxin-producer *A. flavus* S075 was inhibited after 10-14 days by all four selected anti-fungal strains, *i.e. Lb. fermentum* M017, *Lb. fermentum* 223, *H. opuntiae* H17, and *Sacc. cerevisiae* H290, at 51–95% when applied as single cultures and at 100% when the strains were combined into four co-cultures, each composed of a *Lb. fermentum* and one of the two yeast strains. As a conclusion, these four LAB-yeast co-cultures are recommended for future applications to limit the growth of filamentous fungi and the concomitant mycotoxin production during the fermentation of cocoa beans.

1. Introduction

With a global annual production estimated at 4.7 million tonnes in the crop season 2016/2017, cocoa is the main ingredient for chocolate production (Beg et al., 2017; ICCO, 2017). The first step in cocoa production is a spontaneous fermentation process which is necessary to remove the pulp around the beans allowing metabolites to enter the cotyledon and turning the astringent and bitter flavours into precursors of typical chocolate aroma and flavours (De Vuyst and Weckx, 2016). During the fermentation, there is a well-defined microbial succession of yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) (Pereira et al., 2016), however, filamentous fungi can also grow, mainly

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in the well-aerated and cold outer layers of the fermentation mass and during the drying process (Nielsen et al., 2013; Schwan and Fleet, 2014). Contamination with filamentous fungi has been associated with internal development of mould, off-flavours, increased free fatty acid levels, and the production of mycotoxins (Nielsen et al., 2013). Mycotoxins reported to be of major significance in cocoa include aflatoxin B1, associated with *Aspergillus flavus* and *Aspergillus parasiticus*, ochratoxin A (OTA), commonly biosynthesized by *Penicillium* spp. and *Aspergillus ochraceus*, fumonisin, produced by *Fusarium* spp., and citrinin produced by *Penicillium citrinum* (Badrie et al., 2015; Copetti et al., 2014; Nielsen et al., 2013; Sánchez-Hervás et al., 2008). Out of 85 cocoa samples collected during sun drying and 65 samples collected during storage in Brazil, 38% and 32%, respectively, were infected by *A. flavus* and 24% and 14%, respectively, by *A. parasiticus* (Copetti et al., 2011). Aflatoxin B1 was detected in 25% of Brazilian cocoa bean samples at levels of up to 5.9 µg/kg (Maciel et al., 2018) and cocoa products sampled in Canada contained up to 2.6 µg/kg aflatoxin B1 (Turcotte et al., 2013). The European Community (EC) limited the level of aflatoxin B1 in nuts intended for direct human consumption or use as an ingredient in food to 2.0 µg/kg, however, no limit was set for cocoa beans.

Microorganisms are of great interest as biocontrol agents to reduce mould growth and the concomitant contamination with mycotoxins. LAB have been intensively studied as biocontrol agents, such as to retard or eliminate fungal growth based on the production of a wide spectrum of antimicrobial compounds in form of organic acids, low molecular weight compounds, hydroxyl fatty acids, or proteinaceous metabolites, *e.g.* bacteriocins (Peyer et al., 2016). Furthermore, yeasts with antagonistic effects against filamentous fungi are frequently used as biopreservatives to control postharvest diseases on fruits (Spadaro and Droby, 2016). As reviewed by Pereira et al. (2016), efforts in recent years have been made to develop starter cultures for the cocoa bean fermentation, comprising different species of LAB, yeasts, and/or AAB. LAB and yeast strains were recently screened for their ability to inhibit OTA-producing filamentous fungi (de Souza et al., 2017; Essia Ngang et al., 2015; Fossi et al., 2016) with the aim of overcoming problems of mould contamination and mycotoxin accumulation during the cocoa bean fermentation and thereby obtaining a safer end product. However to date, no study has reported anti-fungal cultures to control aflatoxin-, citrinin-, or fumonisin-producing fungal strains.

The aim of this study was to develop well-adapted anti-fungal LAB-yeast co-cultures for the cocoa bean fermentation with a focus on inhibiting aflatoxin-, citrinin-, and fumonisin-producing fungal species. In a multiphasic approach, a large number of cocoa-derived LAB and yeast strains was screened for anti-fungal activity *in vitro* and a selection of strains *in vivo* against filamentous fungal strains producing or potentially producing aflatoxin, citrinin, or fumonisin. To provide cultures with a high survival potential during cocoa bean fermentation conditions, the anti-fungal strains' tolerance towards acid, ethanol, and heat stress and their fermentative potential were assessed *in vitro*.

2. Material and methods

2.1. Microbial strains and filamentous fungal spore production

The microbial strains used in the present study, *i.e.* 362 lactic acid bacteria (LAB) and 384 yeasts, were isolated from cocoa bean fermentation and/or drying processes in Honduras (Romanens et al., 2018; unpublished data), Bolivia and Brazil (Miescher Schwenninger et al., 2016), and Switzerland (unpublished data). The 7 filamentous fungi used as indicator strains originated from Honduras and were retrieved from mould-infested dry cocoa beans, except for *Aspergillus candidus* S010 that was isolated from the wood of a fermentation box and *Aspergillus nidulans* S049 that originated from an on-farm fermentation process. Growth of strains for proliferation and maintenance in the laboratory were also described therein. Antibiotic susceptibility of selected LAB strains was tested by disc diffusion tests according to NCCLS guidelines (Patel et al., 2015). LAB and yeast strains were identified with MALDI-TOF MS prior to this study as described in the above-mentioned publications. In the present work, strains with a MALDI-TOF identification score of 1.700–1.999 were listed according to their identification at genus level and strains with a score of ≥ 2.000 with their identification at species level. LAB strains with a score of < 1.700 were listed as LAB based on their catalase-negative and oxidase-negative behaviour. When LAB and yeast strains were additionally identified through genome sequencing and PCR, respectively, in the present study (Section 2.7), their identities refer to these two methods. Filamentous fungal strains were identified previously by sequencing PCR amplicons of the ITS region as described below in Section 2.7. Throughout this study, fungal species were named according to the taxonomy database of the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/). Microbial strains listed by name, *i.e.* 26 LAB (Supplementary Table S1), 63 yeasts (Supplementary Table S2), and the 7 filamentous fungi mentioned below, are stored in the culture collection of Switzerland (CCOS, Wädenswil, Switzerland).

Spore suspensions of the filamentous fungal indicator strains *Aspergillus candidus* S010, *Aspergillus flavus* S075, *Aspergillus nidulans* S049, *Aspergillus tamarii* S078, *Aspergillus versicolor* S085, *Gibberella moniliformis* S003, and *Penicillium citrinum* S005, were prepared for anti-fungal inhibition tests *in vitro* and *in vivo*. The filamentous fungi were therefore incubated for 5–7 days at 25 °C on Malt Extract Agar (MEA) (1.8% [w/v] malt extract [Biolife], 1.8% [w/v] bacteriological agar), and the spores were harvested with 0.15% (w/v) buffered peptone water (Merck, Darmstadt, Germany). The spore suspensions were mixed with glycerol (AppliChem, Darmstadt, Germany) to a final glycerol concentration of 44% (v/v) and stored at −20 °C. Before usage, the spore concentration was determined by plating appropriate dilutions on MEA.

2.2. Cocoa pulp simulation medium

The cocoa pulp simulation medium developed by Lefeber et al. (2010) was slightly adapted for the experiments with LAB and yeast test strains and was therefore named modified Cocoa Pulp Simulation Medium (mCPSM). Soy peptone was substituted with the more economical meat peptone in view of using mCPSM for industrial-scale culture production. The medium contained 2.5% (w/v) fructose (Carl Roth, Karlsruhe, Germany), 2.5% (w/v) glucose (Sigma-Aldrich, Saint Louis, US), 1% (w/v) citric acid (Carl Roth), 0.5% (w/v) yeast extract (Sigma-Aldrich), 0.5% (w/v) meat peptone (Organotechnie, La Courneuve, France), 0.05% (w/v) magnesium sulphate-heptahydrate (Carl Roth), 0.02% (w/v) manganese sulphate-monohydrate (Sigma-Aldrich), 0.1% (v/v) tween 80 (Sigma-Aldrich) (vol/vol), and 1.5% (w/v) bacteriological agar (Biolife, Milan, Italy) when preparing solid medium. To prevent the Maillard reaction, glucose, fructose, and citric acid were sterilized separately and added to the remaining components after sterilization. The pH of the medium was adjusted with 10 M NaOH solution to 4.0 for liquid medium and to 4.5 when preparing agar plates.

2.3. Mycotoxin production of filamentous fungal strains

The potential of *A. flavus* S075 to produce aflatoxin was assessed genotypically by detecting the genes *aflD*, *aflO* and *aflP* by PCR (Scherm et al., 2005) and phenotypically on yeast extract sucrose agar supplemented with 0.3% β-W7M 1.8-cyclodextrin and 0.6% sodium desoxycholate (YCSD) (Jaimez Ordaz et al., 2003). The potential of *G. moniliformis* S003 to produce fumonisin was determined genotypically by assessing the presence of the genes *fum1* and *fum19* by PCR (López-Errasquín et al., 2007), and *P. citrinum* S005 was tested phenotypically for citrinin production on Coconut Cream Agar (CCA) (Mohamed et al., 2013). Further, *A. flavus* S075, *G. moniliformis* S003, and *P. citrinum*

S005 were tested *in vivo* for mycotoxin production by inoculating samples of 80 g of cocoa beans with 6 log CFU/g of spores and sending the mouldy bean samples, after 11 days of incubation at 25 °C, to the contract laboratory UFAG Laboratorien (Sursee, Switzerland) for mycotoxin analyses.

2.4. Inhibition of filamentous fungi by LAB and yeast strains

2.4.1. In vitro anti-fungal agar spot assay

A modified version of the overlay method of Hassan and Bullerman (2008) was used to assess *in vitro* anti-fungal activity of LAB and yeast strains. LAB were inoculated with sterile toothpicks onto De Man-Rogosa-Sharpe (MRS; Biolife) agar buffered with 0.1 M KH_2PO_4-K_2HPO_4 (pH 6.4) or mCPSM agar and incubated anaerobically at 37 °C for 1 day. The resulting agar plates containing the LAB colonies and non-inoculated MEA or mCPSM agar plates for assays with yeasts were overlaid with 10 ml soft agar supplemented with 4 log CFU/ml filamentous fungal spores and tempered at 50 °C. MEA soft agar with 0.7% (w/v) agar was used for LAB agar spot assays and MEA and mCPSM soft agar containing 1.0% (w/v) agar was used in yeast agar spot assays to overlay MEA and mCPSM agar plates, respectively. Yeast strains were inoculated directly onto the soft-agar layer using sterile toothpicks.

The agar spot assay plates of LAB and yeasts were then incubated aerobically at 25 °C for 3 days, except for plates with spores of *G. moniliformis* S003 that were incubated at room temperature (19–23 °C) and in daylight. Excessive growth of *A. flavus* S075 and *A. tamarii* S078 was slowed down by interrupting the incubation after 30 h by a phase of 15 h at 4 °C before placing the plates back in the incubator.

The inhibition of filamentous fungi was evaluated visually after 3 days of incubation, except for yeasts on mCPSM, for which the zones of inhibition were evaluated after 3, 4, 5, and 7 days and the highest value from the different days was used. To determine a score of inhibition (SI), the distance from the edge of the LAB or the yeast colony to the outer edge of the zone of inhibition was assessed, along with the transparency of the inhibition zone. In terms of LAB, SI were determined by rating the inhibition zones on a scale from 0 to 4, allowing half points: (0) no inhibition, (1) spore formation delayed but no clear zone, (2), fungal growth delayed with a small clear zone around the colony (< 2 mm), (3) fungal growth delayed with a medium-sized clear zone around the colony (2–4 mm), and (4) fungal growth delayed with a large clear zone around colonies (> 4 mm). In the case of yeasts, the zones of inhibition were rated based on the following scale: (0) no inhibition, (1) spore formation delayed with a small turbid zone around the colony (< 1 mm), and (2) spore formation delayed with a medium-sized turbid zone around the colony (2–3 mm). These scores resulting from yeast agar spot assays were multiplied by a factor of 2 if the inhibition zone was semi-clear and a factor of 3 for clear inhibition zones, resulting in SI values of between 0 and 6 for yeasts. Average scores of inhibition ($Ø_{SI}$) were calculated per LAB and yeast strain on MRS and mCPSM or MEA and mCPSM, respectively, averaging the scores of inhibition (SI) obtained for the different tested filamentous fungal indicator strains.

2.4.2. In vivo anti-fungal assay on cocoa beans

The *in vivo* anti-fungal activity of LAB and yeast strains was assessed directly on cocoa beans. LAB and yeast strains were propagated once in mCPSM and inoculated in mCPSM broth, followed by incubation at 37 °C for 15 h for LAB and at 25 °C for 21 h for yeasts. After centrifugation, cell suspensions at defined concentrations were prepared by resuspending the pellet in 0.15% (w/v) buffered peptone water. Twenty grams of cocoa pulp-bean mass, previously extracted under sterile conditions from ripe cocoa fruits and stored at −20 °C, was inoculated with fungal spores at 6 log CFU/g, with LAB cells at 6 log CFU/g and/or with yeast cells at 3 log CFU/g in single culture tests and at 2 log CFU/g in LAB-yeast co-culture tests. The inoculated beans were filled into 10-ml petri dishes and incubated for 4 days at 30 °C. After this first incubation step, beans from LAB single-culture trials were incubated for 6–7 days at room temperature (19–23 °C) in a second incubation step. In a second incubation step during yeast and LAB-yeast co-culture assays, beans were placed on Dichloran Rose Bengal Chloramphenicol (DRBC) agar (Sigma-Aldrich, Saint Louis, USA) as described by Pitt and Hocking (2009) and incubated at 25 °C for 10 days. The inhibition of filamentous fungal growth was evaluated visually as share of non-infested bean surface relative to the total surface at the end of the first and the second incubation steps. Furthermore, beans inoculated with only LAB and/or yeast cells, with filamentous fungal spores alone, or without any added cells or spores were incubated and evaluated in an analogous manner.

At the end of the first phase of incubation, the anti-fungal strains were enumerated on the surface of beans that were only inoculated with cells of LAB and/or yeast strains. Two beans with adhering pulp were mixed manually for 1 min with the same weight of dilution solution (0.1% [w/v] bacteriological peptone [Biolife], 0.85% [w/v] sodium chloride) to obtain a uniform homogenate of pulp and dilution solution. After decimal dilution steps, 20 µl of up to four dilutions were applied on one section of an agar plate followed by holding the plate slantwise for the drops to elongate. The enumeration of yeasts was performed on Yeast Extract Glucose Chloramphenicol (YGC) agar (Biolife) after aerobic incubation at 25 °C for 3 days and the counting of LAB on MRS agar after anaerobic incubation at 37 °C for 2–3 days. The pulp pH was measured with a pH meter (pH-Meter 761 Calimatic, Merck) in the pulp-dilution solution homogenate mentioned above.

2.5. Measuring substrates and metabolites in LAB and yeast fermentation filtrates

Starting from pure colonies on mCPSM agar plates, precultures were prepared by inoculation in mCPSM broth and incubation at 37 °C for 19 h for LAB and at 25 °C for 21 h for yeasts. For the fermentations, 20 ml of mCPSM were inoculated with 1% (v/v) of the preculture and incubated during 48 h at 37 °C for LAB and at 25 °C for yeasts. Two technical replicate fermentations were performed per strain. Samples were collected after 48 h of incubation and centrifuged at 14,000 × *g* for 12 min. The supernatants were diluted 1:5 (vol/vol) with Milli-Q water, filtered (0.2 µm, Sartorius AG, Goettingen, Germany), and stored at −20 °C until analysis. Residual amounts of the substrates glucose, fructose, and citric acid and the amount of produced lactic acid, acetic acid, ethanol, and mannitol were determined in the fermentation samples using high-performance liquid chromatography with refractive index detector (HPLC-RI) according to Romanens et al. (2018). The acidification property of LAB was determined by measuring the pH in samples taken from the LAB cultures after 0 and 48 h with a pH meter (pH-Meter 761 Calimatic, Merck).

2.6. Determining growth of LAB and yeast strains under stress conditions

Growth curves in mCPSM were recorded under optimal reference conditions of LAB at 37 °C and of yeasts at 25 °C and under stress conditions by adding different concentrations of ethanol, lactic acid, and/or acetic acid to mCPSM or by applying increased incubation temperatures (Table 1). After addition of the chemicals, which were purchased from Sigma-Aldrich (Saint Louis, USA), to mCPSM, the medium's pH was readjusted to 4.0 with 10 M NaOH solution. For the precultures, mCPSM was inoculated with pure colonies from mCPSM agar plates and incubated for 5–7 h at 37 °C for LAB and 17–23 h at 25 °C for yeasts. After centrifugation, the pellets were resuspended in fresh mCPSM and diluted to an absorbance value of 1.0 optical densitiy (OD_{600}). One percent (v/v) thereof was inoculated in 250 µl of the respective medium in 96-well plates, leading to an initial absorbance value of 0.01OD_{600}. Growth was monitored by measuring OD_{600} every hour after a shaking step during 48 h in a microplate reader (Biotek Synergy 1, Vermont, US) or a Bioscreen C (Oy Growth Curves Ab Ltd., Helsinki, Finland). Per reference or stress condition-strain combination,

Table 1
Reference and stress conditions applied to test LAB and yeast strains for stress tolerance including weights per stress condition used to calculate the weighted average growth ($Ø_{Growth}$).

	Concentration [%, v/v]					
	[°C]	Eth	LA	AA	Condition	Weight [%]
LAB	37	–	–	–	Reference	–
	37	10	–	–	Single metabolite	10
	37	–	0.7	–	Single metabolite	10
	37	–	–	1.4	Single metabolite	10
	37	3	0.3	0.7	Combined metabolites	35
	45	–	–	–	Temperature	20
	47	–	–	–	Temperature	15
					Sum	100
Yeasts	25	–	–	–	Reference	–
	25	10	–	–	Single metabolite	10
	25	–	1.5	–	Single metabolite	10
	25	–	–	0.7	Single metabolite	5
	25	–	–	1	Single metabolite	5
	25	7	1	0.4	Combined metabolites	35
	45	–	–	–	Temperature	35
					Sum	100

To calculate the $Ø_{Growth}$ for LAB and yeasts, growth in presence of single metabolites was weighted with 30%, in presence of combined metabolites with 35%, and at elevated temperatures with 35% of total weight. Eth = ethanol; LA = lactic acid; AA = acetic acid; – = not applicable.

three biological replicate measurements were performed and growth was calculated by dividing the maximal OD_{600} reached within 48 h of incubation under the stress condition by the maximal OD_{600} reached at the respective reference condition. Per LAB and yeast strain, the weighted average growth ($Ø_{Growth}$) was calculated over all stress conditions using the weights listed in Table 1. The stress factors were weighted according to their importance during cocoa bean fermentations. Growth in presence of single metabolites was weighted with 30%, *i.e.*10% for each single metabolite, growth in presence of combined metabolites with 35%, and growth at elevated temperatures with 35% and subdivisions were made where necessary.

2.7. Analysis of genomic sequences of LAB and yeast strains

Genomic DNA of LAB strains was isolated using a lysozyme-based cell wall digestion step and the Wizard genomic DNA purification kit (Promega, Dübendorf, Switzerland) as described previously (Inglin et al., 2017) and was sequenced using Illumina Miseq 125-bp paired-end sequence technology. The raw reads were imported and paired in the CLC genomics workbench 8.0 and assembled using the following settings: the automatic word and bubble size option was activated and the minimum contig length set at 2000 bp. Contigs were exported as nucleotide FASTA files for further analyses. Protein-encoding sequences (CDSs) were predicted using a heuristic HMM algorithm available at Genmark (http://exon.gatech.edu/GeneMark/). Predicted CDS were compared to the protein version of the Comprehensive Antibiotic Resistance Database (McArthur et al., 2013; https://card.mcmaster.ca/about), version 1.1.1 from 5th October 2016 using basic alignment search tool protein (BLASTP). BLAST was locally performed using the CLC genomic workbench 8.0, using a cut-off value of $e < 10^{-20}$. Virulence factors were identified by BLASTP with the predicted proteome against the full set of proteins from the virulence factor database (http://www.mgc.ac.cn/VFs/, downloaded on 27th October 2016). Cut-off values were identical as for antibiotic resistance identification. LAB strains were identified using a co-occurring k-mers mapping at KmerFinder 2.0 (https://cge.cbs.dtu.dk/services/KmerFinder-2.0/) (Hasman et al., 2014; Larsen et al., 2014).

Yeast strains were identified by sequencing the rRNA gene internal transcribed spacer (ITS) region. Fungal DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep Protocol (Zymo Research Corp.). The ITS region was amplified with ITS1 and ITS4 primers according to Glass and Donaldson (1995) and PCR amplicons were subjected to Sanger sequencing (GATC Biotech, Konstanz, Germany). The sequences were compared with sequences available in the NCBI 18S rRNA genes database using BLAST.

3. Results

3.1. Mycotoxin production by three filamentous fungal strains

Three filamentous fungal strains, *i.e. A. flavus* S075, *G. moniliformis* S003, and *P. citrinum* S005, were tested for their potential to produce mycotoxins on gene level and phenotypically. PCR amplification products for genes responsible for aflatoxin biosynthesis, *i.e. aflD*, *aflO* and *aflP*, were achieved with *A. flavus* S075 and this strain also produced aflatoxin when tested on YCSD agar. However, the concentrations of aflatoxins B1, B2, G1 and G2 were below the limit of detection of 0.5 µg/kg when tested on cocoa beans. The relevant genes for fumonisin production, *fum1* and *fum19*, were found in *G. moniliformis* S003, but when beans were inoculated with this strain, neither fumonisin B1 nor B2 was detected at a limit of detection of 50 µg/kg. *P. citrinum* S005 produced citrinin both on CCA agar and on cocoa beans, and a concentration of 61 mg/kg was measured on the latter.

3.2. Inhibition of filamentous fungi in vitro

Anti-fungal activities *in vitro* of LAB and yeasts as revealed by agar spot assays are demonstrated (Tables 2 and 3) in the following section. In terms of LAB, 362 cocoa-derived strains were tested on buffered MRS against *G. moniliformis* S003 and *P. citrinum* S005 and average scores of inhibition ($Ø_{SI}$) are shown per LAB strain (Table 2). In the genera *Lactobacillus* and *Pediococcus*, 88 out of 244 and 6 out of 46 strains,

Table 2
Average scores of inhibition ($Ø_{SI}$) of 362 LAB strains against *G. moniliformis* S003 and *P. citrinum* S005 revealed by an *in vitro* agar plate assay on buffered MRS agar.

Species	$Ø_{SI}$		
	0.0–1.0	1.1–2.5	2.6–4.0
Enterococcus sp.	4	1	–
Enterococcus casseliflavus	4	–	–
Enterococcus faecalis	1	–	–
Enterococcus faecium	1	2	–
Enterococcus galinarium	1	–	–
***Enterococcus* sp. total**	**11**	**3**	–
Fructobacillus sp.	–	**1**	–
Lactobacillus sp.	21	68	33
Lactobacillus amylovorus	8	3	–
Lactobacillus fermentum	1	9	–
Lactobacillus nagelii	–	2	–
Lactobacillus paraplantarum	–	–	2
Lactobacillus pentosus	–	3	5
Lactobacillus plantarum	–	41	48
***Lactobacillus* sp. total**	**30**	**126**	**88**
Leuconostoc sp.	15	2	–
Leuconostoc pseudomesenteroides	1	1	–
***Leuconostoc* sp. total**	**16**	**3**	–
Pediococcus sp.	6	23	6
Pediococcus acidilactici	3	8	–
***Pediococcus* sp. total**	**9**	**31**	**6**
Weissella sp.	**2**	–	–
Not identified	**11**	**17**	**8**

$Ø_{SI}$ represent mean values of SI against *G. moniliformis* S003 and *P. citrinum* S075, each determined as single replicate. – = not applicable.
If several species of one genus are listed, the total of strains that belong to this genus are indicated in bold.

Table 3
Average scores of inhibition ($Ø_{SI}$) of 384 yeast strains against *G. moniliformis* S003 and *A. flavus* S075 revealed by an *in vitro* agar plate assay on MEA.

Species	$Ø_{SI}$		
	0.0–0.5	1.0–1.5	2.0–6.0
Candida glabrata	–	–	2
Candida parapsilosis	1	2	–
Candida sp. **total**	**1**	**2**	**2**
Diutina rugosa	**–**	**3**	**2**
Hanseniaspora guilliermondii	1	1	–
Hanseniaspora opuntiae	77	9	–
Hanseniaspora sp.	36	7	–
Hanseniaspora sp. **total**	**114**	**17**	–
Meyerozyma guilliermondii	3	–	–
Meyerozyma sp.	1	–	1
Meyerozyma sp. **total**	**4**	–	**1**
Pichia kluyveri	1	–	–
Pichia kudriavzevii	33	87	–
Pichia manshurica	12	9	1
Pichia sp.	14	7	–
Pichia sp. **total**	**60**	**103**	**1**
Rhodotorula glutinis	–	–	1
Rhodotorula sp.	–	1	–
Rhodotorula sp. **total**	–	**1**	**1**
Saccharomyces cerevisiae	5	3	–
Saccharomyces sp.	21	7	3
Saccharomyces sp. **total**	**26**	**10**	**3**
Schwanniomyces etchellsii	**4**	**2**	–
Torulaspora delbruekii	2	–	–
Torulaspora sp.	4	5	3
Torulaspora sp. **total**	**6**	**5**	**3**
Trichosporon asahii	**–**	**3**	**10**

$Ø_{SI}$ represent mean values of SI against *G. moniliformis* S003 and *A. flavus* S075, each determined as single replicate. – = not applicable.
If several species of one genus are listed, the total of strains that belong to this genus are indicated in bold.

respectively, were categorized with high $Ø_{SI}$ values of 2.6–4.0, while 36 strains belonging to the genera *Enterococcus*, *Fructobacillus*, *Leuconostoc*, and *Weissella*, were classified with medium or low $Ø_{SI}$ of 1.1–2.5 or 0.0–1.0, respectively.

To select strains from the 362 LAB strains tested on buffered MRS for anti-fungal *in vitro* screening on mCPSM, 64 LAB strains were preliminarily tested in an agar spot assay on mCPSM. These preliminary tests revealed higher $Ø_{SI}$ on mCPSM for *Lb. fermentum* and *Lb.* sp. than for *Lb. paraplantarum*, *Lb. pentosus*, and *Lb. plantarum* strains (data not shown). Therefore, 26 LAB strains, *i.e.* strains that reached $Ø_{SI} \geq 2.0$ on buffered MRS and had been identified as *Lb. fermentum* or *Lb.* sp. and strains that reached $Ø_{SI} \geq 2.0$ on mCPSM in the preliminary tests, were selected for *in vitro* anti-fungal activity screening on mCPSM against seven filamentous fungal strains (Supplementary Table S1). All 26 LAB strains inhibited the seven tested filamentous fungal strains, *i.e.* displayed $Ø_{SI}$ of at least 1.0 for each individual fungal strain, and reached medium to high $Ø_{SI}$ of 2.3–3.1 (Supplementary Table S1). Over all LAB strains, *A. candidus* S010 was the most sensitive fungal strain inhibited on average with an SI of 3.1 ± 0.4, whereas the most resistant fungus was *A. flavus* S075 inhibited on average with an SI of 1.9 ± 0.4. Exemplary inhibition zones on mCPSM against *P. citrinum* S005 of LAB strains M017 (II), M018 (III), and M031 (VI) with SI of 2.5 are shown in Supplementary Fig. S1a.

In terms of yeasts, 384 strains were tested on MEA against *G. moniliformis* S003 and *A. flavus* S075 and average scores of inhibition ($Ø_{SI}$) are shown per yeast strain (Table 3). The yeast genus *Trichosporon* was the genus with the highest anti-fungal scores on MEA with 100% of strains with $Ø_{SI} \geq 1.0$ and 43% of strains with $Ø_{SI}$ 2.0–6.0. The genera *Pichia* and *Saccharomyces* demonstrated a moderate anti-fungal activity, with 63% and 33% of the strains, respectively, reaching $Ø_{SI} \geq 1.0$, while the genus *Hanseniaspora* showed the lowest anti-fungal activity with only 12% of strains with $Ø_{SI} \geq 1.0$.

From the 384 yeast strains tested on MEA, 63 strains with $Ø_{SI} \geq 1.0$ were selected for *in vitro* agar spot assays on mCPSM against six filamentous fungal strains (Supplementary Table S2). The zones of inhibition for yeast strains were generally smaller and more turbid than for LAB strains (Supplementary Fig. S1b). $Ø_{SI}$ varied considerably for different yeast strains, with values between 0.2 and 2.3, with highest $Ø_{SI}$ on mCPSM found for the *Candida glabrata* strains H30 and H29 with 2.3 and 2.2, respectively (Supplementary Table S2). Most *Hanseniaspora* and *Saccharomyces* strains reached medium $Ø_{SI}$ between 1.0 and 2.0, with *Hanseniaspora opuntiae* H17 and *Saccharomyces cerevisiae* H290 showing $Ø_{SI}$ of 1.8 and 1.5, respectively. Low $Ø_{SI}$ of 0.0–0.9 were detected for most tested strains of the genera *Diutina*, *Meyerozyma*, *Pichia*, *Schwanniomyces*, *Torulaspora*, and *Trichosporon*. The share of filamentous fungal strains inhibited with SI ≥ 0.5 varied from one out of six to six out of six for different yeast strains; yeast strains *Hanseniaspora opuntiae* H17 inhibited five out of six and *Saccharomyces cerevisiae* H290 all six fungal indicator strains on mCPSM.

3.3. Carbon metabolism and acidification properties of anti-fungal LAB and yeast strains

Anti-fungal strains, *i.e.* 26 LAB and 45 yeast strains previously tested for *in vitro* anti-fungal activity on mCPSM (Supplementary Table S1 and S2) were characterized in regards to assimilation of mCPSM's substrates, production of metabolites, and, in the case of LAB, to acidification properties (Supplementary Table S3 and S4). In terms of yeasts, the genera *Torulaspora* and *Trichosporon* were excluded from this test due to safety concerns in view of their application in food fermentation.

The 10 g/l citric acid present in fresh mCPSM was completely metabolized within 48 h by 21 out of 26 LAB strains, including *Lb. fermentum* strain M017, one strain was citrate-negative, and another four strains, amongst them *Lb. fermentum* 223, converted citric acid incompletely (Supplementary Table S3). All tested LAB strains metabolized the sugars glucose and fructose, both present at levels of 25 g/l in fresh mCPSM. Fructose appeared to be converted preferentially, leading to an accumulation of mannitol at concentrations of 10.4–26.4 g/l. Lactic and acetic acids were produced by all 26 tested strains, at concentrations of 6.3–11.2 g/l and 3.3–7.9 g/l, respectively. *Lb. fermentum* strain M017 produced 8.6 g/l lactic acid and 6.2 g/l acetic acid and *Lb. fermentum* 223 10.3 g/l lactic acid and 3.9 g/l acetic acid. The pH measurements showed that the medium's initial pH of 4.0 remained unchanged or was slightly lowered resulting in final pH values of 3.4–4.0 (Supplementary Table S3).

The substrate and metabolite analyses of 45 yeast strains after 48 h revealed that citric acid was metabolized by none of the strains (Supplementary Table S4). Both glucose and fructose were used, glucose being consumed preferentially, to produce ethanol at concentrations of up to 13.2 g/l mainly by yeasts belonging to the genera *Candida*, *Hanseniaspora*, *Meyerozyma*, *Pichia*, and *Saccharomyces*, whereas yeasts of the genus *Diutina* produced no ethanol. *Sacc. cerevisiae* produced 7.4 g/l of ethanol, whereas no ethanol was produced by *H. opuntiae* H17.

3.4. Stress tolerance of anti-fungal LAB and yeast strains

To investigate the physiological adaptation of the 26 anti-fungal LAB and the 45 anti-fungal yeast strains (Section 3.3) to the cocoa bean fermentation environment, their growth was recorded in mCPSM under different stress conditions (Fig. 2; complete data sets in Supplementary Table S3 and S4). Percentage growth refers to OD_{600} measured under the stress condition relative to OD_{600} at the respective reference condition and at a relative growth of ≥10%, the strain was considered as tolerant towards the tested stress condition.

Out of the 26 tested LAB strains, 24 tolerated 10% ethanol, 26 0.7%

lactic acid, 25 1.4% acetic acid, and 22 a combination of 3% ethanol, 0.3% lactic acid, and 0.7% acetic acid (Fig. 2a; Supplementary Table S3). A high temperature tolerance was observed for LAB, with 24 out of 26 strains tolerating 45 °C and four also 47 °C. The two *Lb. fermentum* strains M017 and 223 were tolerant towards all tested conditions except for temperatures of 47 °C and strain M017 generally appeared more stress tolerant than 223, especially towards 10% ethanol and 45 °C, resulting in weighted average growth ($Ø_{Growth}$) of 53% for M017 and 33% for 223 (Supplementary Table S3).

Amongst the 45 yeast strains, the highest tolerance towards 10% ethanol was found for the genus *Saccharomyces*, with growth rates close to 100% for 11 out of 13 strains (Fig. 2c). Furthermore, seven out of nine *Hanseniaspora* strains (Fig. 2b), two out of three *Candida* strains, and all ten tested *Pichia* strains tolerated 10% ethanol, while no ethanol tolerance was recorded for *Diutina*, *Rhodotorula*, and *Schwanniomyces* strains (Supplementary Table S4). All 45 yeast strains tolerated a concentration of 1.5% lactic acid, a great part of the strains with growth values around 100%. Out of the ten tested yeast genera, *Diutina* and *Pichia* showed the hightest acetic acid tolerance, with 14 out of 15 strains growing in the presence of 1.0% acetic acid, followed by the genus *Saccharomyces*, with all strains tolerating 0.7% acetic acid and eight out of 13 strains 1.0% acetic acid. The genus *Hanseniaspora* was one of the less acetic acid tolerant yeast genera, with only five of nine strains tolerating 0.7% acetic acid. Most *Saccharomyces* and *Pichia* strains as well as two of nine *Hanseniaspora* strains tolerated the combined metabolites, *i.e.* 7% ethanol, 1.0% lactic acid, and 0.4% acetic acid. For most yeasts, no growth was observed at elevated temperatures and only the two tested *C. glabrata* and five of ten *Pichia* strains grew at 45 °C and one *Pichia* strain at 47 °C (data not shown). The two yeast strains selected for future applications, *H. opuntiae* H17 and *Sacc. cerevisiae* H290, tolerated all single metabolite stress conditions except for 1.0% acetic acid for H17, and H290 additionally tolerated the presence of combined metabolites, resulting in $Ø_{Growth}$ of 15% for *H. opuntiae* H17 and 43% for *Sacc. cerevisiae* H290 (Supplementary Table S4).

3.5. Inhibition of filamentous fungi in vivo

By means of an anti-fungal assay on cocoa beans, the inhibition capacity of anti-fungal LAB and yeast strains and of LAB-yeast co-cultures was assessed *in vivo* against *A. flavus* S075 and/or *G. moniliformis* S003 and *P. citrinum* S005 (Table 4; Fig. 1). For this test, 14 LAB and 16 yeast strains were chosen with means of $Ø_{Growth}$ and normalized $Ø_{SI}$ on mCPSM of ≥47% for LAB (Supplementary Table S3) and of ≥23% for yeasts (Supplementary Table S4). LAB strain 204 was excluded as it did not tolerate 1.4% acetic acid and yeasts of the genera *Candida* and *Pichia* were excluded due to safety concerns in view of future usage in food applications. Fig. 1 shows exemplarily cocoa pulp-bean mass that had been inoculated with spores of *A. flavus* S075, *G. moniliformis* S003, or *P. citrinum* S005 alone or in combination with cells of the anti-fungal strains *Sacc.* sp. H291 or *Lb. fermentum* M017 or the non-anti-fungal control strains *Lb. fermentum* 193 or *Schwanniomyces etchellsii* H12. In this example, filamentous fungal growth was inhibited at 100% by the anti-fungal strains and at 0% by the non-anti-fungal control strains.

The anti-fungal assays on cocoa beans for LAB and yeasts revealed 98–100% of growth inhibition of *A. flavus* S075 for 6 LAB and 13 yeast strains after 4 days (Table 4). Until the end of the test duration, *i.e.* 10–11 days for LAB and 14 days for yeasts, beans had turned partly mouldy, resulting in 75–100% of growth inhibition for the 6 LAB strains and 0–95% for the 13 yeast strains. Amongst these 6 LAB strains, 5 also inhibited growth of *G. moniliformis* S003 and *P. citrinum* S005 at 100% after 10–11 days (Table 4). *Lb. fermentum* strains M017 and 223 were selected based on 100% growth inhibition of *G. moniliformis* S003 and *P. citrinum* S005 and 88–100% growth inhibition of *A. flavus* S075 after 10–11 days and high stress tolerance, *i.e.* $Ø_{Growth}$ of ≥33% (Supplementary Table S3). *H. opuntiae* H17 was selected for its high growth inhibition capacity of *A. flavus* S075 of 95% after 14 days and its tolerance towards single metabolites and *Sacc. cerevisiae* H290 was chosen for combining a growth inhibition capacity of 51% against *A. flavus* S075 after 14 days with tolerance towards single and combined metabolites (Supplementary Table S4). These selected LAB and yeast strains were combined to four LAB-yeast co-cultures, which completely suppressed growth of *A. flavus* S075 on the bean surface after 14 days. No inhibition was seen for four and incomplete inhibition for one co-culture, in which the yeast and/or the LAB strain was a non-anti-fungal control strain. Cell concentrations on the bean surface after 4 days of incubation were 9–10 log CFU/g for LAB and 5–9 log CFU/g for yeasts when anti-fungal single and co-cultures were inoculated and the pH changes from day 0 to 4 ranged from −0.3 to +1.7 (Table 4).

Table 4
Growth inhibition of aflatoxin-producer *A. flavus* S075 by 14 LAB and 16 yeast strains and 4 LAB-yeast co-cultures as revealed by *in vivo* anti-fungal assays on cocoa beans as well as change in pulp pH and cell counts of inoculated LAB and yeast strains.

Anti-fungal strain or co-culture (no. of biological replicates)		Growth inhibition of *A. flavus* S075 [%]		Pulp pH	Cell count [log CFU/g]	
					MRS	YGC
		t_4	t_{end}	Δ		t_4
Lactic acid bacteria						
M038	LAB (1)	100	100	+0,8	n.d.	–
M089*	*Lb. fermentum* (2)	100	96	−0.3	9	–
222*	*Lb. fermentum* (2)	100	92	−0.2	9	–
223*	***Lb. fermentum* (2)**	**100**	**90**	0.0	9	–
M031*	*Lb. fermentum* (2)	100	75	−0.1	9	–
M017*	***Lb. fermentum* (2)**	**98**	**88**	−0.1	9	–
M080*	*Lb. fermentum* (2)	96	45	+0,1	9	–
M091*	*Lb. fermentum* (2)	90	51	+0,1	9	–
221*	*Lb.* sp. (1)	80	10	−0.1	9	–
M117B*	*Lb. fermentum* (1)	40	0	+0,1	9	–
18*	*Lb.* sp. (1)	25	0	−0.1	9	–
1*	*Lb.* sp. (1)	20	10	0.0	9	–
24	*Lb.* sp. (1)	0	0	+0,6	9	–
M083	*Lb.* sp. (1)	0	0	−0.3	9	–
193[a]	*Lb. fermentum* (4)	0	0	0.0	9	–
Yeasts						
H17	***H. opuntiae* (3)**	**100**	**95**	+0,4	–	6
H362	*Sacc.* sp. (3)	100	91	+0,4	–	8
H369	*H.* sp. (3)	100	88	+0,4	–	8
H358	*Sacc.* sp. (3)	100	79	0.0	–	8
H361	*Sacc.* sp. (3)	100	67	+0,2	–	8
H290	***Sacc. cerevisiae* (3)**	**100**	**51**	+0,2	–	8
H156	*Sacc. cerevisiae* (3)	100	45	+0,4	–	8
H356	*Sacc.* sp. (3)	100	36	+0,1	–	8
H357	*Sacc.* sp. (3)	100	33	+0,1	–	8
H311	*H.* sp. (3)	100	33	+0,1	–	8
H363	*Sacc.* sp. (3)	100	13	+0,3	–	8
H291	*Sacc.* sp. (3)	100	7	+0,1	–	9
H323	*H.* sp. (2)	100	0	+0,4	–	8
H26	*Sacc. cerevisiae* (3)	78	68	+0,1	–	8
H24	*Sacc. cerevisiae* (3)	67	48	+0,4	–	8
H359	*Sacc.* sp. (3)	67	19	+0,1	–	8
H12[a]	*Sch. etchellsii* (3)	0	0	+0,3	–	8
LAB-yeast co-cultures						
***Lb. fermentum* 223 × *H. opuntiae* H17 (1)**		100	100	+0.5	10	6
***Lb. fermentum* 223 × *Sacc. cerevisiae* H290 (1)**		100	100	−0.1	9	8
Lb. fermentum 223 × *Sch. etchellsii* H12[a] (1)		0	0	−0.2	10	5
***Lb. fermentum* M017 × *H. opuntiae* H17 (1)**		100	100	+0.1	9	5

(*continued on next page*)

Table 4 (*continued*)

Anti-fungal strain or co-culture (no. of biological replicates)	Growth inhibition of *A. flavus* S075 [%]		Pulp pH	Cell count [log CFU/g]	
				MRS	YGC
	t_4	t_{end}	Δ		t_4
***Lb. fermentum* M017 × *Sacc. cerevisiae* H290 (1)**	100	100	+0.1	9	8
Lb. fermentum M017 × *Sch. etchellsii* H12[a] (1)	70	0	+0.3	9	4
Lb. fermentum 193[a] × *H. opuntiae* H17 (1)	0	0	0.0	9	<
Lb. fermentum 193[a] × *Sacc. cerevisiae* H290 (1)	0	0	+1.7	9	8
Lb. fermentum 193[a] × *Sch. etchellsii* H12[a] (1)	0	0	+0.8	9	8
Controls					
A. flavus S075	0	0	–	–	–
No cultures	–	–	+0,2	4	<

Beans inoculated only with LAB or yeast strains were used to determine LAB counts on MRS and yeast counts on YGC, respectively, and to measure pulp pH. Anti-fungal strains or co-cultures highlighted in bold were finally selected for future applications.

* LAB strains inhibited growth of *G. moniliformis* S003 and *P. citrinum* S005 at 100% after 10–11 days (data not available for yeasts and LAB-yeast co-cultures); t_4 = day 4; t_{end} = day 10–11 for LAB and day 14 for yeasts and co-cultures; Δ = change in pulp pH from day 0 to 4; – = not applicable; n.d. = not determined; ≤ below detection limit of 2 log CFU/g.

[a] Non-anti-fungal control strains.

3.6. Identity and safety of anti-fungal LAB and yeast strains

LAB and yeast strains providing anti-fungal potential for future applications were identified by means of molecular biological methods. Anti-fungal LAB strains M017, M031, M080, M089, M091, 222, 223 and the non-anti-fungal control strain 193 were identified through genome sequencing as *Lb. fermentum* (Supplementary Table S1). By sequencing the ITS region, the yeast strains H290 and H17 were identified as *Sacc. cerevisiae* and *H. opuntiae*, respectively (Supplementary Table S2). To assess safety criteria of *Lb. fermentum* strains M017 and 223, their genomes were tested for known antibiotic resistance and virulence genes. These revealed, for both strains, a hit for chloramphenicol resistance gene catB10 from *Pseudomonas aeruginosa* with sequence identity of amino acids of 40% and 42%, respectively, but no virulence genes. The resistance could not be confirmed phenotypically in a disc diffusion test. Therefore, *Lb. fermentum* strains M017 and 223 can be considered as safe according to criteria applied by the European Food Safety Authority (EFSA) (EFSA, 2013; Ricci et al., 2017).

4. Discussion

Several studies have suggested that screening on strain level is necessary to select suitable culture strains for the cocoa bean fermentation due to large intraspecies differences (Pereira et al., 2017, 2016). In the current study, a multiphasic approach was applied to select well-adapted anti-fungal LAB and yeast strains for the cocoa bean fermentation from a large number of LAB and yeast strains derived from cocoa bean post-harvest processes.

An important part of this study was the screening for anti-fungal activity, in which LAB and yeasts were tested against up to seven filamentous fungal species that reflect the fungal community of the Honduran cocoa bean fermentation (Freimüller Leischtfeld, unpublished data). The biosynthesis of aflatoxin and citrinin was demonstrated for *A. flavus* S075 and *P. citrinum* S005 on agar plates and cocoa beans, respectively, and genes involved in fumonisin production were detected in the genome of *G. moniliformis* S003.

As a first step in the anti-fungal screening process of LAB and yeast strains, an agar spot assay was applied as a high-throughput method to

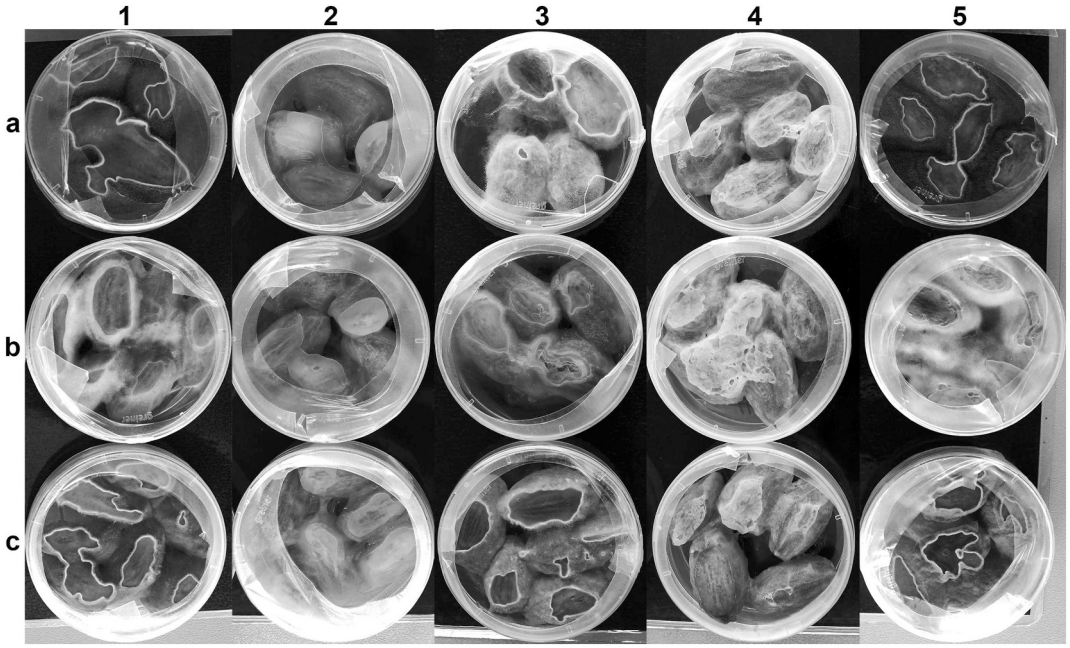

Fig. 1. *In vivo* anti-fungal cocoa bean assay to assess growth inhibition capacity of anti-fungal LAB and yeast strains against fungal indicator strains *A. flavus* S075 (a), *G. moniliformis* S003 (b), and *P. citrinum* S005 (c). Column 1 shows the control with only filamentous fungi inoculated. Beans in columns 2, 3, 4, and 5 were additionally inoculated with anti-fungal *Lb. fermentum* M017, non-anti-fungal *Lb. fermentum* 193, anti-fungal *Sacc. cerevisiae* H290, and non-anti-fungal *Sch. etchellsii* H12, respectively. After 4 days of incubation at 30 °C, growth of 100% by all three filamentous fungal strains is visible in columns 1, 3, and 5, whereas in columns 2 and 4, the anti-fungal strains *Lb. fermentum* M017 and *Sacc. cerevisiae* H290, respectively, suppressed the indicator strains resulting in 0% fungal growth, *i.e.* 100% growth inhibition. For better visibility of filamentous fungal growth, the contrast of the picture was increased. A whitish appearance of the beans in column 4 was caused by the growth of *Sacc. cerevisiae* H290.

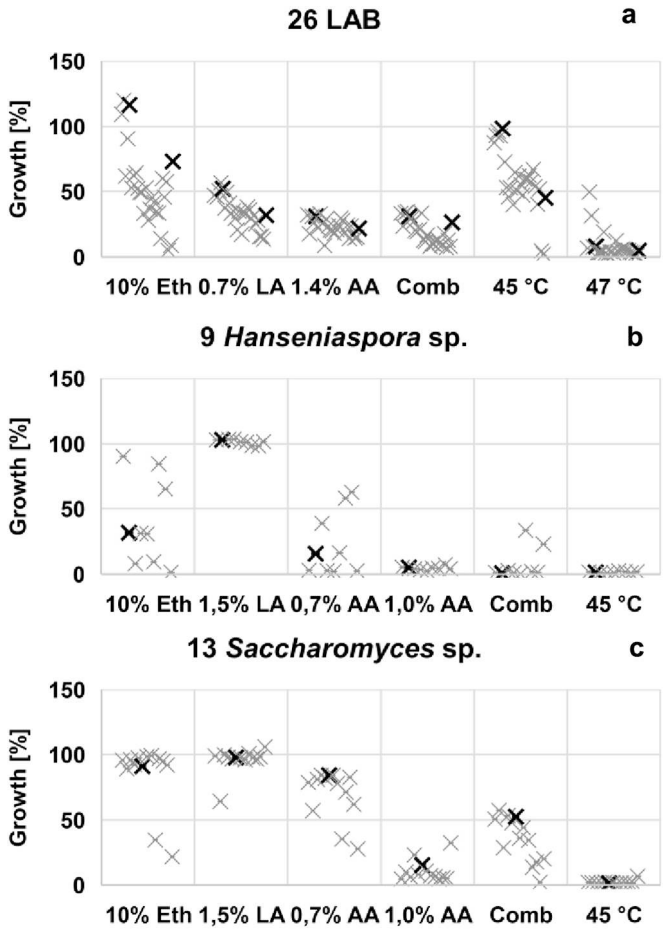

Fig. 2. Growth determined as OD_{600} of 26 LAB strains (a), 9 *Hanseniaspora* sp. (b), and 13 *Saccharomyces* sp. (c) under different stress conditions relative to OD_{600} measured at the respective reference conditions. Different positions along the x-axis help to discriminate between single strains and have no further meaning. Black crosses highlight strains *Lb. fermentum* M017 (a; left), *Lb. fermentum* 223 (a; right), *H. opuntiae* H17 (b), and *Sacc. cerevisiae* H290 (c). Eth = ethanol; LA = lactic acid; AA = acetic acid; Comb = combination of 3% ethanol, 0.3% lactic acid, and 0.7% acetic acid (a) or 7% ethanol, 1.0% lactic acid, and 0.4% acetic acid (b and c).

preselect strains with anti-fungal activity. For this test, two different growth media were used, which greatly affected the resulting anti-fungal scores in the case of LAB and to a lesser extent for yeasts. *Lb. plantarum*, as well as *Lb. pentosus* and *Lb. paraplantarum*, which are closely related to *Lb. plantarum* according to Stiles and Holzapfel (1997), showed high scores of inhibition on buffered MRS but not on mCPSM agar, whereas *Lb. fermentum* was the main anti-fungal LAB species on mCPSM. A possible reason for this might be the different metabolisms of the two LAB species, as *Lb. fermentum* strains produce more acetic acid and *Lb. plantarum* more lactic acid (Adler et al., 2013; Lefeber et al., 2011). The difference in pH of the growth media, 6.4 in buffered MRS and 4.5 in mCPSM, might have affected the inhibitory effect of these acids as described by Copetti et al. (2012); or as mentioned in the review of Dalié et al. (2010), pH and/or nutrient composition of the media could have influenced the production of anti-fungal substances. It is assumed that mCPSM is more suitable to select anti-fungal LAB and yeast strains for cocoa bean fermentation, as the pH and the composition of mCPSM are similar to the ones of cocoa pulp in contrast to MRS with a higher pH and a different nutrient composition. Finally, the screening on mCPSM agar against seven filamentous fungal species, five *Aspergillus* spp., one *Penicillium* spp., and one *Gibberella* spp., revealed broadband anti-fungal activity for the tested LAB and yeast strains, indicating that the anti-fungal strains used in the present study might also inhibit other filamentous fungi, *e.g.* OTA-producing strains.

In a final step, a cocoa bean assay specifically designed for this study was applied to screen LAB and yeast single and co-cultures at smallest scale using 20 g of cocoa-pulp bean mass. The anti-fungal activity previously observed *in vitro* on mCPSM was confirmed *in vivo* for LAB, *Saccharomyces*, and *Hanseniaspora* strains with up to 100% growth inhibition of *A. flavus* S075 on the bean surface for 4 days and for most LAB strains with 100% growth inhibition of *G. moniliformis* S003 and *P. citrinum* S005 during 14 days. *A. flavus* S075 showed a higher inhibition resistance against LAB strains in this test and *in vitro* on mCPSM compared to *G. moniliformis* S003 and *P. citrinum* S005 and was therefore used as indicator strain to assess the anti-fungal activity of yeast strains and LAB-yeast co-cultures. By combining anti-fungal LAB and yeast strains into co-cultures, complete growth inhibition of *A. flavus* S075 was prolonged to 14 days, compared to 4 days for LAB and yeast single cultures, indicating an interaction of LAB and yeast strains in anti-fungal activity mechanisms.

In terms of anti-fungal mechanisms, there was no evidence that a change in pH was responsible for the anti-fungal activity of LAB strains, indicating that other mechanisms are involved in the suppression of filamentous fungi, such as the secretion of anti-fungal compounds, *e.g.* organic acids, cyclic dipeptides, fatty acids, or proteinaceous compounds (Brosnan et al., 2014; Crowley et al., 2013; Miescher Schwenninger et al., 2008). Surprising were the low concentrations of 2 and 3 log CFU/g of yeast cells in single and co-culture tests that sufficed to inactivate 6 log CFU/g filamentous fungal spores, compared to 6 log CFU/g of inoculate in the case of LAB. It indicates that the yeasts used different mechanisms than the LAB to prevent filamentous fungal growth, such as in the competition for limiting nutrients and space or the production of lytic enzymes (Spadaro and Droby, 2016). Anti-fungal mechanisms responsible for the inhibitory activity of the LAB and yeast strains remain known.

To assess the anti-fungal LAB and yeast strains' fermentative potential, concentrations of main substrates and metabolites were determined after fermentation in mCPSM. The breakdown of citric acid that was observed for most tested LAB strains, is ascribed to the use of citric acid as a co-substrate in heterolactate fermentation by citrate-positive LAB species, as described by De Vuyst and Weckx (2016), and is an important role of LAB in the cocoa bean fermentation (Ouattara et al., 2017). Most tested LAB strains were more efficient in converting citric acid than sugars similarly to the findings of Pereira et al. (2012). The tested LAB species consumed both glucose and fructose, fructose being consumed almost completely and converted mostly into mannitol, as described previously by Adler et al. (2013). The tested LAB strains, *i.e.* 24 *Lb. fermentum* and 2 not further identified LAB strains, produced lactic and acetic acid. The final concentrations of 6–11 g/l and 3–8 g/l were in a similar range to the amounts of 6–13 g/l and 5–11 g/l of lactic and acetic acid, respectively, that were measured for cocoa-derived *Lb. fermentum* strains under comparable conditions (Adler et al., 2013; Lefeber et al. 2011, 2010; Pereira et al. 2012). A moderate lactic acid production is an important characteristic for potential culture strains, as excessive amounts of the non-volatile acid can negatively affect the fermented beans' acidity according to Lefeber et al. (2010). In contrast, acetic acid has been described as being important as its diffusion into the beans, together with ethanol and heat, is responsible for the death of the embryo and drives biochemical conversions inside the cotyledon (De Vuyst and Weckx 2016; Ozturk and Young 2017).

All tested yeast strains were citrate negative, which is in accordance with the reports of Fernández Maura et al. (2016) and Pereira et al. (2012), who did not find citrate conversion amongst various tested cocoa-derived yeast strains. Conversely, other studies described citrate conversion for cocoa-derived yeast strains, *P. kudriavzevii* and *Candida* sp. being the most often mentioned yeast species with this characteristic

(Daniel et al. 2009; Samagaci et al. 2016). The majority of the yeast strains converted the sugars glucose and fructose simultaneously, glucose being used in slightly higher amounts than fructose, which has been described previously by Pereira et al. (2012). The reason for poor or non-existent ethanol production of some tested yeast strains might be the lack of fresh medium caused by not shaking the fermentation tubes. Higher ethanol levels were observed when some of the strains were fermented in continuously shaken tubes, *e.g.* 18.4 g/l for the strain *H. opuntiae* H17 (data not shown). A tendency for higher ethanol production was observed for yeasts with higher $Ø_{SI}$ on mCPSM agar and higher $Ø_{Growth}$ in mCPSM, *i.e.* strains of the genera *Candida*, *Hanseniaspora*, *Pichia*, and *Saccharomyces*, which might be ascribed to a well-adapted metabolism of these yeasts to mCPSM.

Concurrently, anti-fungal LAB and yeast strains were screened for their physiological adaptation to the cocoa bean fermentation environment by measuring growth in mCPSM under different stress conditions. For that, conditions commonly occurring during the fermentation of cocoa beans were chosen, such as high ethanol and acid levels and elevated temperatures. At the time point of maximal LAB counts, up to 1–6% ethanol, 0.1–1.7% lactic acid, 0.1–2.0% acetic acid, and temperatures of up to 35–48 °C have been reported in the cocoa pulp-bean mass (Camu et al. 2007; Lagunes Gálvez et al. 2007; Nielsen et al. 2007; Papalexandratou et al. 2013, 2011; Romanens et al. 2018). LAB strains tested in the present study were highly tolerant towards ethanol and heat and to a lesser extent tolerant towards acetic acid, with a majority of the strains tolerating 10% ethanol, 1.4% acetic acid, and 45 °C and some strains even 47 °C. This might explain why LAB persist until the end of the fermentation process, when temperatures and acetic acid concentrations increase (De Vuyst and Weckx 2016). The LAB strains' lactic acid tolerance was rather low with a concentration of 0.7% lactic acid reducing growth by > 50% on average. A low lactic acid tolerance could be a main limiting factor for LAB growth in the fermentation process and is in accordance with Visintin et al. (2016), who reported lactic acid to be stressful towards cocoa-derived LAB strains.

When yeast cell concentrations are at their maximum during spontaneous cocoa bean fermentations, metabolite levels of up to 1–6% ethanol, 0.1–1.5% lactic acid, and 0.0–0.4% acetic acid, and maximal temperatures of 28–30 °C have been measured (Camu et al. 2007; Lagunes Gálvez et al. 2007; Nielsen et al. 2007; Papalexandratou et al. 2013, 2011; Romanens et al. 2018). Similarly to LAB, yeast strains showed a high ethanol tolerance, with most strains growing in the presence of 10% ethanol. The genera *Saccharomyces* and *Pichia* showed the highest growth rates, coinciding with Daniel et al. (2009), De Vuyst and Weckx (2016), Fernández Maura et al. (2016), and Pereira et al. (2012), who ascribed ethanol tolerance to cocoa-derived *Sacc. cerevisiae* and *P. kudriavzevii* strains. In contrast to LAB, yeasts showed a high tolerance towards lactic acid, with all tested strains growing at 1.5% lactic acid and the growth of *Pichia* strains even seeming to be favoured by lactic acid, as more than half of the tested *Pichia* strains grew better in the presence of lactic acid than under reference conditions. The tolerance towards acetic acid and elevated temperatures, however, was low amongst yeasts, which, as stated by Daniel et al. (2009), explains the disappearance of yeast populations when temperature and acetic acid increase at the end of the cocoa bean fermentation. Despite the generally low acetic acid and heat tolerance of cocoa-derived yeast strains, the tolerance of several yeast strains towards 0.7% or even 1.0% of acetic acid and 45 °C shows the potential of these strains to survive conditions occurring during the yeast phase as specified above.

Generally, the tolerance of LAB and yeast strains towards elevated concentrations of metabolites was greatly reduced when ethanol, lactic acid, and acetic acid were combined. Similar findings have been reported by Samagaci et al. (2014), who found that elevated temperature combined with ethanol, lactic acid, or acetic acid, was a main stress hindering the growth of yeast strains in the cocoa bean fermentation. In terms of yeasts, strains of the genera *Pichia* and *Saccharomyces* were best adapted to combined metabolite stress.

Based on the analysis of anti-fungal activity *in vivo*, carbon metabolism and stress tolerance, four anti-fungal LAB-yeast co-cultures, each composed of one LAB and one yeast strain, were selected for future applications as protective cultures in the cocoa bean fermentation. The safety of the selected *Lb. fermentum* strains in view of their application in a food product was approved by confirming the absence of virulence factors and functional antibiotic resistance determinants. The chloramphenicol resistance gene that was found was not functional according to the phenotypic resistance assays. In terms of yeasts, strains of *H. opuntiae* and *Sacc. cerevisiae* were selected due to the safe use of these genera in food products, as illustrated by the qualified presumption of safety (QPS) status attributed to *Sacc. cerevisiae* and *H. uvarum* by EFSA (Ricci et al. 2017). Furthermore, the selected yeast species are known to play an important role in the cocoa bean fermentation process (De Vuyst and Weckx 2016; Fernández Maura et al. 2016; Papalexandratou et al. 2013). Strains of the species *P. kudriavzevii*, which were excluded due to safety aspects, might – if their safety for the cocoa-consumer can be approved – be used as protective cultures due to their high tolerance towards stress conditions. This species has been described as a good aroma producer, by Pereira et al. (2017), and as being well-adapted to the cocoa bean environment (Samagaci et al. 2016); furthermore *Pichia* sp. with high inhibitory effect against OTA producing filamentous fungi were reported by de Souza et al. (2017).

5. Conclusion

The multiphasic screening approach performed in this study with modified cocoa pulp simulation medium was demonstrated to be a suitable method to select anti-fungal LAB-yeast co-cultures for the cocoa bean fermentation. Four co-cultures were finally selected, each composed of a *Lb. fermentum* strain, M017 or 223, in combination with either *S. cerevisiae* H290 or *H. opuntiae* H17, which inhibited at 100% the growth of aflatoxin-producer *A. flavus* S075 on the surface of cocoa beans. The adaptation of the single strains to the cocoa bean fermentation, *i.e.* the ability of the LAB strains to metabolize citric acid and to grow under ethanol, acid, and heat stress and the yeasts ability to perform an alcoholic fermentation and to tolerate high ethanol and lactic acid levels, suggests their potential to participate in the LAB and the yeast phase of cocoa bean fermentation, respectively. Further trials should include the application of the anti-fungal LAB-yeast co-cultures to the cocoa bean fermentation to assess their influence on the fermentation process and the quality of resulting dry cocoa beans in terms of fermentation degree and sensorial characteristics and to evaluate the co-cultures' efficiency in reducing the number of mouldy beans and the levels of mycotoxins.

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Competing interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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