International Journal of Food Microbiology 306 (2019) 108258

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

The preservative propionic acid differentially affects survival of conidia and germ tubes of feed spoilage fungi

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ARTICLE INFO

Keywords:
Feed
Xerophiles
Aspergillus
Penicillium
Fusarium
Propionic acid
Conidia
Germ tube

ABSTRACT

Propionic acid is widely used as a preservative in (poultry) feed. In this study we have isolated and identified fungal strains from nine samples poultry feed originating from different countries. The majority of the strains were Aspergilli with a eurotium-morph, such as *Aspergillus proliferans* and *A. chevalieri*. These and three other species were selected and tested for their sensitivity towards the feed preservative propionic acid, among them *Penicillium lanosocoeruleum*. The determined MIC values of 6.1–31 mM of these poultry feed specific fungi were well in the range as described in literature. Propionic acid (at 31 mM) damages conidia (spores) in a species dependent fashion after a 24-hour-treatment. The majority of the conidia (over 70%) of *P. lanosocoeruleum* germinated within 60 h on agar medium, while 50 and 80% of the *A. chevalieri* and *A. proliferans* conidia did not, respectively. Dependent on the species, cell damage was visible after incubation with propionic acid. Germ tubes of *P. lanosocoeruleum* in a biofilm showed extensive (85%) cell death after a 30 min treatment with propionic acid and slightly lower sensitivity was observed with *A. proliferans* (62% cell death). Microscopic analysis of these fungal biofilms revealed extensive damage to the cell membrane and showed distorted intracellular structures. Fluorescent life-dead staining of the germ tubes showed a clear dose response of propionic acid indicating a fungicidal effect on these growing cells. These results show that conidia can be inactivated by propionic acid, but that germ tubes show a much higher sensitivity. These observations shed new light on the mode of action of this important preservative to prevent fungal contamination of feed.

1. Introduction

Animal feed products often are maintained at a relatively low water activity (< 0.8) that prevents growth of the majority of bacterial and fungal spoilers. However, a number of so-called xerophilic (dry-loving) fungi, such as *Aspergillus* species (with a eurotium-morph, the sexual state) and *Wallemia sebi* (Zajc and Gunde-Zimmerman, 2018) are surprisingly well adapted to growth under these conditions and, as such, shorten the shelf life of feed. Fungal growth on feed decreases its nutritional value, and results in the accumulation of off-flavours and/or secondary metabolites such as mycotoxins that affect the animal health and performance (Vesonder et al., 1988). Mycotoxin contamination causes billions of dollars in losses worldwide due to reduced crop yields, lost trade revenues (local and international), livestock illnesses, and adverse human health effects. To ensure the safety and stability of food- and feed products, agents are added that slow down or prevent the development of these fungi (Brul and Coote, 1999).

Organic acids and their salts are globally used in animal nutrition for microbial preservation and supporting animal health. They have been proven effective in maintaining animal growth performance and can increase body weight, improve feed conversion ratio and reduce colonization of pathogens (as *Salmonella*) in the intestine (Khan and Iqbal, 2016; Lückstädt and Mellor, 2011; Van Immerseel et al., 2006). Therefore, they are regarded as a natural alternative for the use of antibiotics as growth promotors, which has been banned in the EU since 2006 (Castañon, 2007).

The weak organic acid propionic acid is an important preservative in food and feed and inhibits growth of various spoilage bacteria, yeasts and fungi (Haque et al., 2009; Stratford et al., 2013), including the mycotoxigenic fungi *Aspergillus flavus* and *A. parasiticus* (Holmquist et al., 1983). Propionic acid is more lipophilic compared to acetic acid and has properties of a (volatile) fatty acid. It has been generally accepted that the acid can cross the plasma membrane in the undissociated form and causes acidification in the cytoplasm of the living cell leading to energy depletion and slow growth. The acid has a pKa of 4.88, which means that half of the acid is in the undissociated form at

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https://doi.org/10.1016/j.ijfoodmicro.2019.108258
Received 18 February 2019; Received in revised form 20 June 2019; Accepted 28 June 2019

this pH. In the yeast *Saccharomyces cerevisiae*, the acid may enter the cell via non-facilitated diffusion and induces the zinc-finger transcription factor War1p that leads to the expression of the ATP-binding cassette (ABC) transporter Pdr12p (for a review see, Mollapour et al., 2008) that is able to transport the preservative anion outside the cell.

The effective antifungal activity (MIC, minimal inhibitory concentration) of propionic acid against several fungi is typically in the range of 7.8 to 39 mM (0.1 to 0.5%, w/v), when grown on Sabouraud broth at a pH of 5. These include several *Aspergillus* and *Penicillium* species (Haque et al., 2009). Dagnas et al. (2015) reported MIC values, based on germination of conidia within 35 days, from fungi isolated from spoiled bakery products. The values, as calculated, using mathematical modelling, ranged from 8.7 mM for *Aspergillus pseudoglaucus* (*Eurotium repens*), 18.8 mM for *Aspergillus niger* and 19.4 mM for *Penicillium corylophilum*.

In this study we have evoked fungal growth from nine poultry feed samples by means of a shelf life test, and isolated and identified fungal strains for further study. A selection of strains was tested for the MIC of propionic acid in several fungi-specific growth media. The damage of propionic acid to conidia and germ tubes was evaluated. For the latter, a novel test was developed using biofilms of germ tubes and the fluorescent dye TOTO-1. The results show that conidia and germ tubes are damaged by propionic acid, but to different extents.

2. Material and methods

2.1. Enumeration of fungi on nine samples of poultry feed

Fungi were isolated from nine poultry feed samples that were subjected to a shelf life test, that is in use in practice to predict the microbial stability of a feed product. In short, an amount of 250 g of feed was transferred into a 1 L plastic beaker. A smaller beaker, containing 60 mL water, was placed in the middle of the feed sample. The closed beaker was incubated at 37 °C until visible fungal growth occurred on the feed. The time recorded of the onset of fungal growth is a measure for the expected shelf life of the (untreated) feed. The feed samples examined in this study originate from 4 countries on 4 continents (see Table 1).

2.2. Isolation and identification

Initially, a series of photographic portraits were made of the samples, followed by a detailed inspection with a stereomicroscope (NIKON SM Z25). For this, a sample feed was taken with a spatulum, and the material transferred carefully to an empty Petri-dish for examination by stereo microscopy. Micrographs were taken at different depths of field and subsequently stacked in Adobe Photoshop resulting in one picture with optimal definition.

Strains were isolated from samples of contaminated feed. For this, a sample of feed was transferred into an empty Petri-dish and small pieces of fungal material were collected from the surface of the feed by means of a preparation needle. In addition, fungal material and small kernels were taken from deeper levels of the sample, where fungal development was not clearly visible (although some aerial hyphae were observed between kernels). Fungal samples were plated out on DG18 medium, which contains 220 $g.L^{-1}$ glycerol in order to lower the water activity of the medium (to 0.95) to support growth of xerophilic fungi. After 10 to 12 days of cultivation at 25 °C, these plates were used to isolate fungi for pure cultures. Conidia of 51 different pure cultures were mixed in 30% (w/v) glycerol and kept in vials at −80 °C.

Table 1
Samples of poultry feed used in this study.

Sample	Type	Country
1	Layer mash	Zimbabwe
2	Layer mash	Zimbabwe
3	Layer mash	Zimbabwe
4	Layer mash	Zimbabwe
5	Poultry feed	Indonesia
6	Poultry feed	Indonesia
7	Broiler feed	Guatemala
8	Layer feed	The Netherlands
9	Poultry feed	The Netherlands

A selection of 8 phenotypically different strains was identified using a sequence-based approach. Genomic DNA was isolated and a part of the β-tubulin gene (*BenA*) and the calmodulin gene (*CaM*) were amplified and sequenced for the *Aspergillus* and *Penicillium* isolates; a part of the translation elongation factor 1α (*Tef1*) was sequenced for identification of the *Fusarium* isolate. DNA, PCR and sequencing were according the methods described in Samson et al. (2010). The generated sequences were compared with other (reference) sequences present in GenBank and internal databases of the Westerdijk Institute using BLAST.

2.3. Minimally inhibitory concentration of propionic acid for the different feed-borne isolates

Spores were obtained from *Aspergillus chevalieri*, *A. proliferans*, *Penicillium lanosocoeruleum*, *Fusarium equiseti* species complex and *Wallemia sebi* after incubation for 7 days on malt extract agar (CM 0059, Oxoid Ltd, Basingstoke, UK) supplemented with 0.01 $g.L^{-1}$ $ZnSO_{4}.7H_2O$ and 0.005 $g.L^{-1}$ $CuSO_4.5H_2O$ (Samson et al., 2010); DG18 (CM 0729, Oxoid Ltd) and/or malt extract agar supplemented with 20% (w/v) sucrose (abbreviated to ME20S, Samson et al., 2010). Conidia were harvested in ACES-buffer (N-(2-Acetamido)-2-aminoethanesulfonic acid, 10 mM, pH 6.8, Merck (Sigma), Darmstadt, Germany), filtered over sterile glass wool and stored on ice until the start of the experiments (Van Leeuwen et al., 2013a). The spores were counted in a haemocytometer (Bürker-Türk, VWR, Amsterdam, The Netherlands), diluted in double strength malt extract broth and added to the wells of a microplate in a volume of 100 μL. Propionic acid was applied in a two-fold serial dilution with concentrations ranging from 0.38 mM to 77 mM. The MIC values, as defined by complete inhibition of growth, were determined by visual inspection of the wells of the microtiter plates after 6 and 14 days of incubation at 25 °C.

2.4. Viability count of conidia

Conidia harvested from cultures of three strains, *Aspergillus chevalieri*, *A. proliferans*, and *P. lanosocoeruleum* grown on DG18 (*Aspergillus*) and MEA medium (*Penicillium*) for 8 days at 25 °C were used to assess if viability of the spores had decreased after weak acid treatment.

Conidia were harvested as described above and counted by means of a Coulter Counter (Multisizer 3, Beckman Coulter, Indianapolis, USA). Conidial density was set to 10^5 cells/ml in 10 ml sterile ACES-buffer in 50 ml Erlenmeyers and incubated with and without 31 mM propionic acid for 24 h at 25 °C under continuous slow shaking (60 r.p.m.). The pH of ACES buffer after addition of propionic acid was 4,1. After incubation, the conidia were diluted 50 times in buffer and a volume of 100 μL was spread plated on DG18 agar in duplo aiming at an inoculation of 200 spores per plate. Simultaneously, conidia were observed with differential contrast microscopy using a Zeiss Axioskop 2 plus (Carl Zeiss AG, Oberkochen, Germany). Erlenmeyers were prepared in duplo. The agar plates were checked visually and colonies were counted after 48 and 86 h.

2.5. Assay of cell damage on germ tubes

To evaluate if damage is afflicted to germ tubes, a novel test was developed in which biofilms of the fungi *A. chevalieri* and *P. lanosocoeruleum* were used. Small 1 cm^2 blocks of colonies of a 1-week-old sporulating culture grown on solid medium were placed in malt extract supplemented with 20% (w/v) sucrose and vortexed at maximal speed to bring the conidia into suspension. The solution was filtered over sterile glass wool and the concentration of conidia was evaluated. A quantity of 1.25 mL medium containing approximately 10^6 spores was placed in a small Erlenmeyer flask (25 mL) and incubated overnight as standing cultures at 25 °C.

A volume of 1.25 mL of propionic acid solutions of 7.7, 15, 23, 31, 46, 62 mM (equivalent to 0.1, 0.2, 0.3, 0.4, 0.6, 0.8% v/v) prepared in ME20S was gently added to the standing cultures and mixed carefully resulting in final concentrations of 3.4 to 31 mM. This culture was incubated for 30 min at room temperature, and biofilm was loosened by means of a pipetting tip and 10 µL culture was added to 90 µL demi water containing 4 µM TOTO-1 (Quinolinium, 1-1′-[1,3-propanediylbis[(dimethyliminio)-3,1-propanediyl]]bis [4-[(3-methyl-2(3H)-benzothiazolylidene) methyl]]-tetraiodide, Thermo Fisher Scientific, Waltham, MA, USA) as a final concentration. After 15 min staining, microscopical slides were prepared for fluorescence microscopy (Zeis Axioskop, Zeiss, Oberkochen, Germany) equipped with Filterblock II (09), 450–490 nm, FT 510, LP 520. Micrographs were taken with the Axiocam software (Zeiss, Oberkochen, Germany). The total time of exposition to propionic acid was 30 min and germ tubes in diluted solutions were counted afterwards in a time window of 30 min starting 15 min after the beginning of staining.

3. Results

3.1. Fungal growth on poultry feed samples

In our study nine different samples of poultry feed originating from 4 countries within 4 continents (see Table 1) were subjected to a shelf life test, with elevated temperature and relative humidity. This accelerated shelf life test is used in practice to quickly access the level of fungal contamination of the feed and to develop preventive steps to secure the microbiological quality of the feed. All feed samples developed visible fungal growth in such a test.

Fig. 1A shows an overview of an example of feed originating from Zimbabwe within the beaker of the shelf life test and the hole in the middle is where the beaker with water was positioned. Fungal growth is visible as green patches, which are visible in an enlargement of the image shown in Fig. 1B. Subsequently, a subsample feed was studied in detail using a stereo microscope. Fig. 1C shows a stacked image showing the composition of the feed containing particles of different structure and color. Especially at the upper (air) surface of the sample a fungal hyphal network is visible and green pigmented fungal spores are formed. Fig. 2 shows details of the aerial hyphae of the fungi and shows smaller and larger spore forming structures. Based on microscopic evaluation of all 9 contaminated feed samples, it was clear that the majority of the fungi belong to the genus *Aspergillus*.

The samples originating from Indonesia showed a different fungal development. These samples contain solely corn kernels and show abundant fungal growth after the shelf life test (Fig. 3A). Different shades of green are visible, indicating the presence of different fungal species that have developed on the feed. Fig. 3B shows details of these fungi and indicate the presence of a third white fungus. In Fig. 3C individual spore-forming structures are visible of two different species.

Sample 8 originates from broiler feed from Guatemala and had a very compact structure and yellow color (Supplementary Fig. 1A). Details of fungal development on the surface of this sample are shown in Supplementary Fig. 1B. The last two samples are from the Netherlands and more comparable to the samples from Zimbabwe (Fig. 1) and characterized by a very loose structure. From each sample, small pieces of fungal material were transferred to DG18 for the isolation and subsequent identification of the isolates. Supplementary Fig. 2 shows examples of such plates.

Fig. 1. Overview of a sample layer mash from Zimbabwe with development of fungi after a shelf life test. A.) The beaker in which the shelf life test is done containing the feed on which patches of fungal growth are visible (black asterisk) B.) The same area as in A.), but enlarged. The arrow shows green conidiophores of an *Aspergillus* species. C.) Detailed view with stereo microscope showing the particles in the feed in detail. The black fat arrow shows the boundary between the top layer and the material within the sample, where it is cut. The white thin arrow shows the cobweb of fungal hypha growing over the top layer. Many conidia-forming structures are visible. Bars are 1 cm (A.), 2.5 mm (B.) and 1 mm (C.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

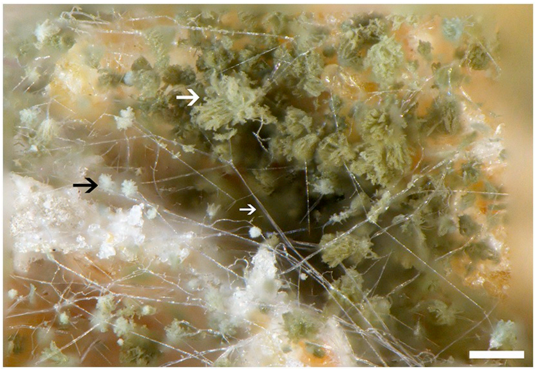

Fig. 2. Detailed and stacked micrograph of the same sample, showing aerial hyphae (small white arrow), mature green conidiophores (fat white arrow) and white immature conidiophores (black arrow) of *Aspergillus* species. Bar = 200 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Isolation and identification of strains

A number of 51 cultures have been stored in the working collection of the Westerdijk Fungal Biodiversity Institute. Of all strains obtained, 92% belong to the genus *Aspergillus* (eurotium-morph). A subselection of 8 isolates consisting of 6 *Aspergillus*, one *Penicillium* and one *Fusarium*

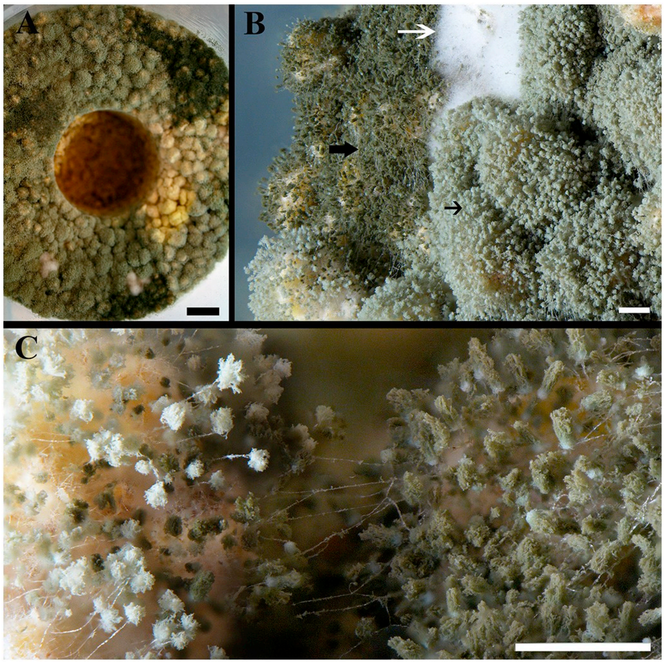

Fig. 3. A.) Poultry feed from Indonesia showing large yellow kernels and abundant growth of several species of fungus. B.) Detail of overgrown kernels with three different species of fungus that from more pigmented dark green conidia (fat black arrow) and lighter grey green pigmented conidia (thin black arrow). The white arrow shows outgrowth of a white mycelium. C.) Conidiophores with light pigmented conidia and dark pigmented conidia (right). The rows of these conidia are more slender/columnar, while the rows of conidia on the left conidiophores are extending more spherical. Bars are 1 cm (A.), 1 mm (B. and C.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

strain, preliminary typed by morphology, were subjected to further identification by means of DNA sequencing and database comparison. These strains were deposited in the Westerdijk culture collection (see Table 2).

Strains CBS 145381, CBS 145383, CBS 145387 and 145388 were identified as *A. chevalieri*. This fungus is formerly known as *Eurotium chevalieri* and is well-known for its yellow-green colored colonies consisting out of green conidiophores (forming conidia) and yellow ascomata (fruiting bodies) that contain numerous asci, each forming 8 ascospores. The strain CBS 145384 was identified as *Aspergillus proliferans*. This fungus formerly known as *Eurotium acutum* is characterized by the formation of yellow/red pigmented aerial hyphae and fruiting bodies containing ascospores. The strain CBS 145382 was identified as a recently described new species, *Aspergillus aurantiacoflavus* very closely related to *A. proliferans* (Chen et al., 2017). As the strain CBS 145384 was originating from poultry feed from Zimbabwe and CBS 145382 is originating from Guatemala, this difference may indicate that these two spoilage strains might indeed be present in the feed at the country of origin. The four *A. chevalieri* strains, however, had identical sequences for both genes.

The *Penicillium* strain was identified as *P. lanosocoeruleum* and the *Fusarium* strain as one belonging to the *Fusarium equiseti* species complex. These isolates are of minor importance as judged by their relative low predominance, but selected to evaluate the sensitivity for preservatives in a broader panel of relevant species originating from feed.

The *Wallemia sebi* strain (DTO 347-D6) has not been subjected to DNA sequencing as it grows very slowly and can be identified based on its typical morphology.

From these strains, a panel of 5 fungal strains was selected for further testing. The panel consists of *A. chevalieri* (CBS 145381) and *A. proliferans* (CBS 145384), *P. lanosocoeruleum* (CBS 145386), *W. sebi*, and the strain belonging to the *F. equiseti* complex (CBS 145385).

3.3. Determination of minimal inhibitory concentration of propionic acid

The minimally inhibitory concentration (MIC) of propionic acid was assessed for 5 selected fungal strains (Table 3). Fungal spores are used as an inoculum in the MIC test. As the stress tolerance of fungal conidia is dependent on environmental conditions during cultivation (Rangel et al., 2015; Hagiwara et al., 2017) we decided to test several growth media for fungi to evaluate if MIC values in general were within the limits observed in literature. We also tested germination and outgrowth in different MIC tests containing different growth media, for those fungal species that could perform growth at lower water activity.

The *Fusarium* strain was grown on malt extract agar (MEA) and DG18 medium and conidia were harvested and used in the microtiter assay with different concentrations of propionic acid in malt extract broth (MEB). MIC values between 6.1 and 15 mM were observed for *F. equiseti*. The same procedure was followed for *P. lanosocoeruleum* with the exception that the MIC for propionic acid was also studied in malt extract supplemented with sucrose (to lower the water activity). *P. lanosocoeruleum* showed MIC values for propionic acid of 6.1–15 mM.

W. sebi and *A. chevalieri* were grown on MEA and DG18 and conidia tested in malt extract supplemented with sucrose exhibit MIC values of 7.7 (*W. sebi*) and 15 mM (*A. proliferans*) propionic acid.

For *A. chevalieri* MIC values for propionic acid were in the range between 7.7 and 31 mM. Conidia from MEA20S plates had a MIC value of 7.7 mM in MEB, whereas conidia from a DG18 plate had a MIC value of 31 mM in MEB supplemented with sucrose. These observations indicate that variation in MIC values possibly occur due to different growth conditions. The MIC values measured were consistent up to at least 14 days of growth.

For all strains, the concentration of propionic acid that resulted in growth inhibiting effects was within a range of 3.1–9.6 mM.

The pH of malt extract broth was measured as 4.77; the pH of a 31 mM propionic acid solution was 3.96.

Table 2
Fungal species identified and used in this study.

Selected strain	Sample No.	Species	Country	Strain no
1	7	*Aspergillus chevalieri*	Guatemala	CBS 145381
2	7	*Aspergillus aurantiacoflavus*	Guatemala	CBS 145382
3	7	*Aspergillus chevalieri*	Guatemala	CBS 145383
4	1	*Aspergillus proliferans*	Zimbabwe	CBS 145384
5	3	*Aspergillus chevalieri*	Zimbabwe	CBS 145388
6	3	*Aspergillus chevalieri*	Zimbabwe	CBS 145387
7	2	*Penicillium lanosocoeruleum*	Zimbabwe	CBS 145386
8	1	*Fusarium equiseti sp. complex*	Zimbabwe	CBS 145385
9	1	*Wallemia sebi*[a]	Zimbabwe	DTO 347-D6

[a] This species was not used for DNA sequence analysis, but identified by its morphological characteristics.

Table 3
MIC values of propionic acid for 5 different fungal species precultured in various media.

Species	Microtiterplate				
	MEB			MEB20S	
	*	**	***	*	***
Aspergillus chevalieri					
MIC (mM)[§]	19	7.7	15	31	31
Aspergillus proliferans					
MIC (mM)				15	15
Fusarium equiseti sp. complex					
MIC (mM)	6.1		15		
Penicillium lanosocoeruleum					
MIC (mM)	6.1		15	15	15
Wallemia sebi					
MIC (mM)				7.7	7.7

Light shading (*) Pre-cultured on malt extract agar. Moderate shading (**) Pre-cultured on malt extract agar supplemented with 20% sucrose (M20S). Darker shading (***) Pre-cultured on DG18 agar medium. [§]The relation between % propionic acid and mM is as follows; 0.04% = 3.1 mM; 0.08% = 6.2 mM; 0.2%= 16 mM; 0.4% = 31 mM. The composition of the media is described in Samson et al. (2010). pH and α_w are for MEA (5,4 ± 0.2, > 0.99 respectively); for M20S (5.4 ± 0.2, 0.99 resp.); for DG18 (5.6 ± 0.955 resp.).

3.4. Viability of conidia after treatment with propionic acid

Table 4 shows the data of the viable count of conidia of three fungal species, *A. chevalieri* CBS 145381, *A. proliferans* CBS 14584 and *P. lanosocoeruleum* CBS 145386 treated with 31 mM propionic acid in ACES buffer for 24 h and subsequently plated out on DG18 medium. If all conidia would germinate and form a colony, a number of 200 colonies was expected. Supplementation of ACES buffer with 31 mM propionic acid resulted in a pH of 4.1. Freshly harvested conidia of the three strains in buffer exhibited the same germination efficiency compared to conidia that were shaken in buffer without propionic acid at 25 °C for 24 h. Light microscopy showed that no germ tubes were formed after this period with all three species. After treatment of conidia with propionic acid, a significant decrease of germination in comparison with the control was observed that was dependent on the species observed. For *A. chevalieri*, an 80% decrease in germination rate was found, but for *A. proliferans* and *P. lanosocoeruleum* this was 49% and 28%, respectively. Light microscopy of treated conidia confirmed cell damage in the case of the *Aspergillus* species, and some changes in the appearance of the cytoplasm within conidia of *P. lanosocoeruleum* (Supplementary Figs. 3–5).

Table 4
Germination efficiency of conidia of fungal strains after treatment with 31 mM of propionic acid for 24 h.

	Number of colonies		
	Upon harvesting[a]	Control[b]	Propionic acid[b]
Aspergillus chevalieri	236 ± 16	230 ± 13	48 ± 7
Aspergillus proliferans	193 ± 10	190 ± 21	97 ± 32
Penicillium lanosocoeruleum	171 ± 10	169 ± 7	123 ± 19

[a] The numbers of colonies of conidia inoculated directly after harvesting.

[b] Colonies counted after 24 h incubation in ACES-buffer with and without propionic acid at 60 rpm at 25 °C. Numbers based on two independent experiments in Erlenmeyers, each inoculated in duplo.

3.5. Microscopic evaluation of damage to germ tubes after treatment with propionic acid

Biofilms of *A. chevalieri* CBS 145381 and *P. lanosocoeruleum* CBS 145386 were prepared in a thin layer of growth medium in an Erlenmeyer flask at rest at 25 °C. Conidia were germinated overnight and both species had formed numerous germ tubes that together formed a thin biofilm at the bottom of the Erlenmeyer flask. The length of the germ tubes, formed overnight, of *A. chevalieri* was markedly shorter compared to *P. lanosocoeruleum* indicating a faster germination and growth for the latter.

Fig. 4A shows control untreated hyphae from *A. chevalieri*. The hyphae are wide, show septa, branches and cell organelles as vacuoles as visible with phase contrast microscopy (top panel). These cells lack fluorescence upon staining with TOTO-1, indicating that the membranes are not damaged (Fig. 4A, bottom panel). After a 30 min incubation with 31 mM propionic acid, hyphae were clearly distorted (Fig. 4B and C, top panels), thinner than compared to untreated cells, and seemed to accumulate light dispersing material inside the hyphae (see arrowhead, Fig. 4B, bottom panel). Hyphae were stained by TOTO-1 (Fig. 4B, C, bottom panels), most prominently the nuclei, which indicates a loss of the plasma membrane barrier function. In contrast, no staining of the spores was found, suggesting that TOTO-1 can enter these cells less effective, even when they are damaged.

Similar observations were done with the fungus *P. lanosocoeruleum*, which had formed a more prominent biofilm, due to faster growth of the germ tubes. Similar to *A. chevalieri*, untreated hyphae showed integrity and details (septa, organelles) with phase contrast microscopy (Fig. 5A, left panel), while thin, distorted hyphae were visible after incubation in 31 mM propionic acid containing medium (Fig. 5B, left panel). Light-dispersing cell contents were visible in many hyphae. Fig. 5B (right panel) shows the effect of propionic acid on TOTO-1 staining of hyphae; many cells and nuclei were stained. Some minor staining of hyphae was observed in the control experiment (Fig. 5A, right panel).

Staining of hyphae was quantified by counting the number of stained hyphae as a function of propionic acid concentration in the medium. Between 80 and 140 hyphae were evaluated by microscopy in each incubation with propionic acid. For both fungal species a clear

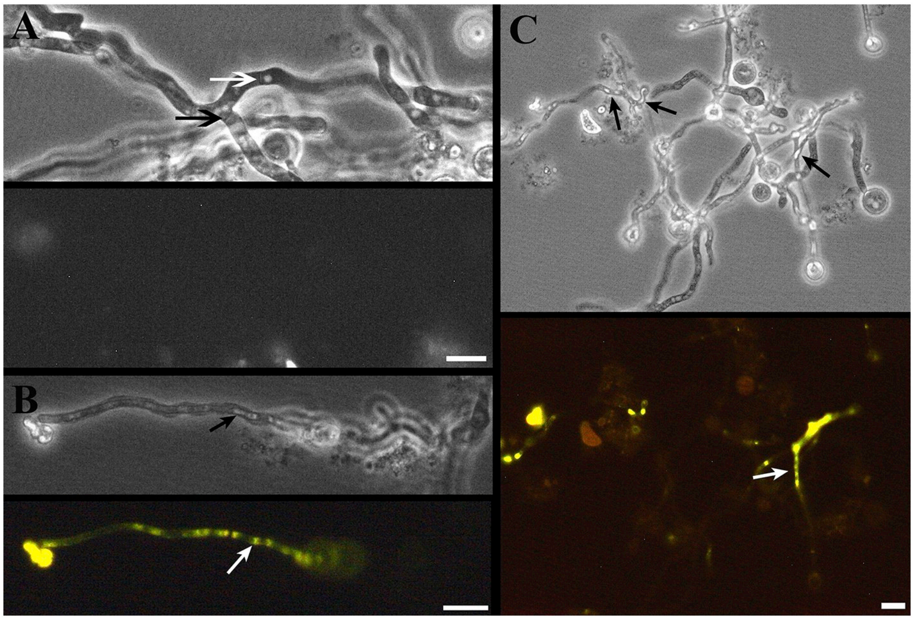

Fig. 4. Effect of 31 mM propionic acid on germ tubes of *Aspergillus chevalieri* (CBS 145381) observed with phase contrast microscopy (left panel) and stained with TOTO-1 and observed with fluorescence microscopy. A.) Non-treated cells show cellular integrity, with septa (black arrow) and cell organelles (white arrow). No staining at all is observed in these cells after minimally 15 min of staining. B.) Germ tube after treatment with 31 mM propionic acid in medium, shows a thin hyphae and abberant structures in the hyphae (black arrow) and staining of nuclei (white arrow) with the fluorescent dye. C.) Germinating spores (round cells) with germ tubes. Many cells are damaged (black arrows, compared to A.) of which a number show staining (white arrow). Bars = 10 μm.

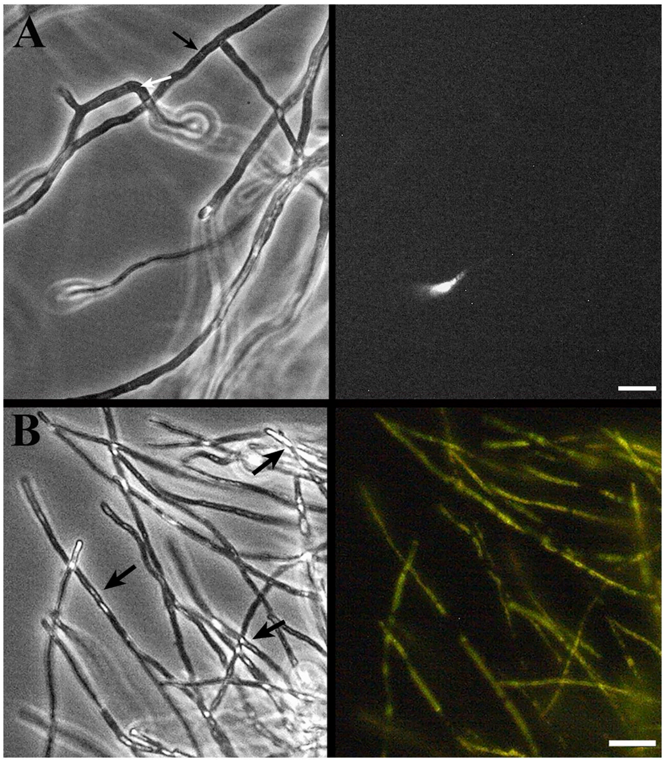

Fig. 5. Effect of 31 mM propionic acid on germ tubes of *Penicillium lanosocoeruleum* (CBS 145386) observed with phase contrast microscopy (left panel) and stained with TOTO-1 and observed with fluorescence microscopy (right panel). A.) Non-treated cells show septa (black arrow) and subcellular organelles (white arrow) visible with phase contrast microscopy. Fluorescence microscopy reveals low staining (see right panel). B.) Long germ tubes after treatment with 31 mM propionic acid in medium, shows thinner hyphae and accumulation of light dispersing material (black arrows). In the right panel, numerous hyphae are stained with the fluorescent dye. Bars = 10 μm.

Table 5
Percentage of germ tubes stained with TOTO-1 of *Aspergillus chevalieri* and *Penicillium lanosocoeruleum* in the presence of propionic acid.

Treatment	No staining	Staining	Total	% Staining
Aspergillus chevalieri				
Control	87	0	87	0
3.8 mM	96	0	96	0
7.7 mM	84	3	87	3.4
11.5 mM	51	31	82	38
15.3 mM	51	22	73	30
21 mM	60	35	95	37
31 mM	42	69	111	62
Penicillium lanosocoeruleum				
Control	134	9	143	6.3
3.8 mM	125	7	132	5.3
7.7 mM	89	43	132	33
11.5 mM	21	83	104	80
15.3 mM	58	80	138	58
21 mM	19	83	102	81
31 mM	15	83	98	85

dose-response of propionic acid was observed (Table 5). Some effects of propionic acid were already visible at a concentration of 7.7 mM and marked effects were visible at 11.5 mM propionic acid and higher. Some damage to hyphae of *P. lanosocoeruleum* in the control experiment was observed due to handling of the fragile hyphae before fluorescent staining. Nevertheless, the increase of cell damage was dramatic for *P. lanosocoeruleum* with 81 and 85% stained hyphae at 21 and 31 mM propionic acid respectively (Table 5). *A. chevalieri* formed less extended germ tubes within the time of incubation and showed a lower numbers of cells stained up to 62% at 31 mM propionic acid.

4. Discussion

In this study we have isolated xerophilic Aspergilli with high predominance from spoiled poultry feed. We assessed the sensitivity of a panel of isolated fungi for propionic acid and evaluated the viability of treated conidia and germ tubes.

The MIC-value of propionic acid for these strains was in the range of 4.6 and 31 mM under conditions where approx. 50–90% of the acid was in the undissociated state and in media with different water activity. This is based on the pK_a of propionic acid (4.9) and the pH of the supplemented growth medium, which is 3.8 in the case of 31 mM propionic acid (4.8 in the absence of the compound). Nine of the 15 measurements (including 4 fungal species) show a MIC of 15–19 mM (Table 3). This MIC-value fits well with earlier data obtained for filamentous fungi, which ranges from 7.2 to 39 mM (Dagnas et al., 2015; Haque et al., 2009; Higgins and Brinkhaus, 1999), but also is similar to that observed with the bacterium *Listeria monocytogenes* (Wemmenhove et al., 2016). Interestingly, the concentration of propionic acid generally applied in feed products is between 15 and 30 mM propionic acid. The dosage of applied propionic acid required to reduce ochratoxin A formation or to prevent aflatoxin formation in a study that addressed damp stored grain was higher, namely between 42 and 100 mM (Scudamore et al., 2004).

However, there are also much higher MIC values reported for propionic acid in both germinating conidia and vegetative yeast cells. Yeast cells from *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* show MIC values of 60 and 170 mM propionic acid at pH 4.0 respectively (Stratford et al., 2013). Kalai et al. (2017) reported a MIC of germinating conidia of the fungi *Penicillium camemberti* and *P. roqueforti* of 200 and 800 mM (at 25 °C, $a_w = 0.99$, pH 5.6), respectively, based on the formation of germ tubes as observed by light microscopy. As calculated from the Henderson-Hasselbalch equation, the concentration of undissociated acid would be 31 and 120 mM respectively, at the pH of measurement.

Our data show that propionic acid affects both conidia and germ tubes, but to different extents. Conidia are added to medium in a MIC test and if outgrowth is not observed at a certain propionic acid concentration, this could be caused by an effect on the spores themselves or on the germination of these cells. The latter involves swelling of the spore and the onset of germ tube formation. Precisely in which stage outgrowth is prevented in a MIC test is not clear. In our experiments, there is evidence for cell damage by propionic acid to conidia when they are exposed to 31 mM of the acid at pH 4.1, dependent on the tested species (between 20 and 80% inactivation).

Dormant (resting) conidia are survival and dispersal structures, metabolically not very active (Novodvorska et al., 2016) and less sensitive towards antifungal compounds (Russell et al., 1975). It is unknown if the conidia, that have been incubated in buffer for 24 h, are in precisely the same physiological state as directly upon harvesting. For example, conidia of *Penicillium rubens* and *Cladosporium halotolerans* can proceed into germination in pure water (Segers et al., 2017); conidia of *Aspergillus niger*, however, need the presence of nutrient molecules for swelling and germ tube formation (Hayer et al., 2014, 2013). In our experiment, no germ tube formation was observed after incubation in buffer while the viability of these cells was unchanged, but sensitivity of these cells could have been increased in time.

Our data also show that there are clear differences between conidia (spores) of different fungal species. This is confirmed in other studies; water-dispersed hyaline (micro) conidia as those produced by *Fusariumoxysporum* were more sensitive to the antifungal compound natamycin in comparison with the airborne conidia of *Penicillium discolor* and *A. niger* (Van Leeuwen et al., 2010, 2013b). During the process of germination, conidia show a decrease in stress resistance. Conidia of *A. fumigatus* become 20–40 times more sensitive for the antifungal amphotericin B methyl ester during germination (Russell et al., 1975). Expression of genes encoding for proteins involved in protection of the cell such as chaperones as well as radical- and peroxide scavengers, dramatically reduce during the first 2 h of germination in conidia of *A. niger* (Van Leeuwen et al., 2013a). Similarly, the level of protecting compatible solutes drops strongly during these stages (Van Leeuwen et al., 2013b).

Our work shows that a biofilm containing growing germ tubes was markedly more sensitive to propionic acid as a 30 min treatment with 31 mM was enough to inactivate over 80% of germ tubes of *P. lanosocoeruleum*, while 20% of the incubated conidia did not show outgrowth upon a 24 h treatment with the acid. For, *A. proliferans* these values were, 62 and 50% respectively. Both fungi showed a clear dose-response to propionic acid as characterized by the number of cells stained with TOTO-1 and the distorted appearance of the cell. Cell damage was observed at a concentration of 8 mM propionic acid or higher. Interestingly, the faster growing *P. lanosocoeruleum* that showed more abundant germination of conidia after acid treatment, was more sensitive towards propionic acid.

The MIC values of the yeasts *Z. bailii* and *S. cerevisiae* reduce with increasing length of the carbon chain of several organic acids while the lipid solubility of the molecule (in the membrane) increases (Stratford et al., 2009, 2013). In the case of both yeast species, resistance against weak acids is conveyed by a small slow-growing subpopulation of cells with acidified cytoplasm resulting in a strongly reduced uptake of the weak acids (Fernández-Niño et al., 2015; Stratford et al., 2013, 2014). The question arises if the intracellular pH of conidia is also lower compared to the values observed in growing hyphae and as such render these cells less susceptible to accumulation of weak acids. There are indications that the pH_{in} in conidia increases during germination in the case of the fungi *Penicillium paneum* as well as *Fusarium culmorum* increase during germination (Chitarra et al., 2005a, 2005b). Care has to be taken here, as in *S. cerevisiae* cells it has been observed that the pH_{in} (or pH_c) is markedly more variable as has been always assumed (Orij et al., 2012). Interestingly, a relationship between higher intracellular pH and growth rate is observed, indicating that growing cells have a higher pH and subsequently are more prone to suffer extensive accumulation of weak acid molecules. Swollen conidia of *A. niger*, close to germ tube formation, at 5 h after the onset of germination exhibit an average pH_{in} of 7.0, but with approx. 1 pH unit variation possible (Stratford et al., 2009).

The organisation of the growing hyphal tip is highly dedicated to ensure a strongly localized tubular extension of the cell and metabolism is intense. These might be factors that are related to the higher sensitivity of these cells for propionic acid. Parton et al. (1997) studied the effect of the release of 50 mM sodium propionate at pH 7.0 near a fast growing hyphal tip of the fungus *Neurospora crassa*. They observed a lowering of the internal pH (as measured by means of a fluorescent dye), a stoppage of growth for a number of minutes followed by the formation of a new hyphal tip, which was thinner and had a lower growth rate. The effective concentration of effective undissociated propionic acid must be low (0.4 mM) at this pH. Another experiment at pH 6.0 (3.5 mM undissociated acid) resulted in a larger acidification without tip regeneration. Dagnas et al. (2015) reported that propionic acid has no effect on radial extension of colonies of fungi on agar surfaces, which may suggest that swelling and germ-tube formation of conidia are sensitive for the weak organic acid, but outgrowth of mycelium is hardly affected.

Could other ways that propionic acid is affecting the growing fungal cell exist? It is known that many fungi can use propionic acid as carbon- and energy source (Fleck and Brock, 2008). In *A. nidulans* the growth inhibitory effect of propionate was shown to be correlated with an increase in intracellular concentration of propionyl-CoA, which strongly inhibits enzymes involved in glucose metabolism: i.e. the pyruvate dehydrogenase complex, succinyl-CoA synthetase and ATP citrate lyase, leading to retardation of fungal growth (Brock and Buckel, 2004). However, in another study evidence was provided of the involvement of an acyl-CoA:carboxylate-transferase in propionyl-CoA detoxification (Fleck and Brock, 2008).

Several studies mention a lower activity of polyketide synthesis (Zhang et al., 2004). The presence of calcium propionate in broiler feed, tested at water activities of 0.85, 0.90 and 0.95, lowered, but did not prevent the presence of aflatoxin (Alam et al., 2014). Holmquist et al. (1983) show that *Aflavus* and *A. parasiticus* were inhibited by 1000 ppm propionic acid (7.8 mM) for over 90% after 30 days at 27 °C, 0.99 water activity and pH 3. The fungi were grown on Sabouraud agar and the number of spores used to inoculate the plates was not specified. The effect of propionic acid was reduced at pH 5 (between 20 and 30% inhibition) and pH 7 (approx. 10% inhibition compared to pH 3). On treated yellow dent corn supplemented with 5000 ppm (38 mM) propionic acid, the fungi were not visible after 30 days, at 27 °C at a moisture content of 16%, but at 23% very little growth was observed in the case of *A. flavus*. Untreated samples showed abundant growth of the fungi.

Our method of gentle administering the weak acid to growing fungal hyphae in medium illustrated that a strong reaction of the germ tubes was evoked by only small amounts of acid in relatively short time, but that the spores are little affected. This could indicate that the weak acid provokes a regulated- or programmed cell death (Carmona-Gutierrez et al., 2018). This is suggested by studies of Yun and Lee (2016) for propionic acid and Guerreiro et al. (2016) for acetic acid. The first authors evaluated the effect of propionic acid on cells of *Candida albicans* and describe apoptotic effects on these cells at a concentration of 100 μM of the weak acid. This is very low compared to our MIC values and shows that many questions still have to be addressed here.

Summarized, these results suggest that growth inhibition of fungi by propionic acid is mainly fungicidal in the case of growing germ tubes due to damage of the cell membrane and the internal integrity of the germ tubes, but that also conidia are partly inactivated, albeit that longer incubation times are needed.

5. Conclusions

Propionic acid has a fungicidal effect on germ tubes and to a lesser extent on conidia. It has to be expected that propionic acid exhibit both fungistatic and fungicidic effects on the same fungus as some populations of conidia (from *P. lanosocoeruleum*) are only inactivated in lower numbers, but will not germinate at that concentration (i.e. have a lower MIC). It is of interest to evaluate the sensitivity of conidia of fungi for the weak acid that are more similar to vegetative cells, such as *F. oxysporum* or *Verticillium fungicola*, which could be useful to prevent plant- or fungal diseases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108258.

Declaration of Competing Interest

None known.

Acknowledgements

This research was funded by Trouw Nutrition. We thank Ralph Litjens, Pim van Kleinhoven, Danielle Smeitink (Trouw Nutrition, Tilburg, The Netherlands) and Gertien Smits (University of Amsterdam) for valuable discussions. We thank Bart Kraak for inoculation of the strains during several experiments. We also thank the reviewers of this manuscript for their comments.

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