A novel antifungal property for the *Bacillus licheniformis* ComX pheromone and its possible role in inter-kingdom cross-talk

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Abstract

Quorum sensing molecules (QSMs) regulate, through a chemical communication process, multiple complex systems in bacterial and some fungal populations on the basis of cell-density. The bacterial QSMs involved in inter-kingdom cross-talk may exhibit antagonistic activity against fungi. This provides an important opportunity for bio-control of fungal invasion in plants. It has been shown that cultures of *Bacillus* spp. inhibit fungal growth. Here, we explore the inhibitory potential of the industrial workhorse *Bacillus licheniformis* NCIMB-8874 and its QSM (ComX pheromone) on the growth of *Aspergillus flavus*, a cereal, legume and nut crop pathogen. Our studies show that ComX filtered extracts from cultures of *B. licheniformis* can cause a significant reduction in the growth of *A. flavus* NRRL 3357 and ESP 15 at a concentration as low as 13 $\mu g ml^{-1}$. This work evidences, for the first time, the inter-kingdom utility of the bacterial quorum sensing ComX pheromone indicating potential antifungal food security against *A. flavus*.

Key words

Quorum sensing molecule, *Bacillus*, *Aspergillus*, natural antifungal, fungal colony area, inter-kingdom communication

Introduction

Quorum Sensing (QS) is an intra- and interspecies microbial communication process based on cell-density found in bacteria and fungi (Nealson *et al*. 1970; Fuque *et al*. 1994; Hogan 2006). In many bacterial species, more than one class of compound engage in the network of regulatory systems controlling the response of a bacterial population to environmental changes, by regulating diverse genes (Atkinson and Williams 2009). QS mechanisms in bacteria are often classified into three different cell communication systems operating in i) Gram-negative bacteria, ii) Gram-positive bacteria, and iii) both groups of bacteria as a universal QS system (Miller and Bassler 2001). In filamentous fungi, when QSMs of e.g. *Aspergillus terreus*, *A. flavus* or *A. nidulans* are exogenously added to their own fungal cultures, they effect morphological changes as well as secondary metabolite production such as lovastatin, penicillin and aflatoxin (Schimmel *et al*. 1998; Brown *et al*. 2008 and 2009; Williams *et al*. 2012).

Inter-kingdom chemical signalling involved in parasitic and symbiotic relationships with unicellular and complex eukaryotes is mediated through the cross-sensing of pheromone and pheromone-like compounds, including QSMs (Fox 2004; Rumbaugh 2007; Mullard 2009). These communication networks are also subject to significant plasticity on account of enzymatic modification and degradation of the signalling molecules involved. For instance, one of the bacterial QSMs, Acyl-Homoserine Lactone (AHL), which directs bacterial compound synthesis, is also recognised by animal, plant, seaweed and fungal cells (Dudler and Eberl 2006). Animal cell gene expression in response to QSMs has been reported to be altered in an immune modulatory fashion (Sperandio 2003; Shiner *et al*. 2005). In green seaweed, Joint *et al*. (2002) demonstrated that *Enteromorpha* zoospores are attracted by synthetic AHLs to settle on bacterial biofilm microcolonies in an AHL concentration-dependent manner. In higher plants, the *Medicago truncatula* legume (Mathesius *et al*. 2003) and the tomato rhizosphere (Schuhegger *et al*. 2006) have also been reported to respond to bacterial AHL signalling. In the legumes, the concentration of more than 150 proteins is altered, whereas AHL in tomato rhizospheres increases salicylic acid concentration in the plants' leaves, thereby enhancing systemic resistance against the fungal leaf pathogen *Alternaria alternate*. The latter evidence suggests that AHLs play an

important role in the bio-control activity of rhizobacteria (Mathesius *et al*. 2003; Schuhegger *et al*. 2006).

On the other hand, several reports indicate that fungi interfere with bacterial QS by producing AHL antagonists (Rasmussen *et al*. 2005). Plant defence signals such as salicylic acid have been reported to up-regulate the expression of an AHL-degrading enzyme in *Agrobacterium tumefaciens*. Alternatively, AHL-antagonists are produced by plants and fungi to block bacterial QS (Bauer and Mathesius 2004; Dudler and Eberl 2006; Williams 2007). Elsewhere, it has been reported that the *Candida albicans* QSM compound, farnesol, suppresses transcription of the *Pseudomonas aeruginosa* quinolone signal (PQS) QSM (Cugini *et al*. 2007). Interestingly, Lee and colleagues (Lee *et al*. 2015) recently reported that farnesol can also block eukaryotic signalling pathways (e.g. STAT3) in mice, potentially exerting, anti-proliferation and pro-apoptotic activities on tumours.

Inter-kingdom communication also underpins food security through regulation of the metabolism of fungi with pathogenic and saprophytic potential in agricultural crops. *Aspergillus* species are common soil saprophytes found worldwide, responsible for pre-harvest and post-harvest disease in several agricultural seed crops (Amaike and Keller 2011). Among these fungi, *A. flavus* and *A. parasiticus* are the predominant species responsible for aflatoxin contamination of crops prior to harvest or during storage (Miller 1995; Yu *et al*. 2004; Gokmen *et al*. 2005). At the same time, *A. fumigatus* and *A. flavus* are the primary causes of mould aspergillosis in man. Thus, respiratory (Dufresne *et al*. 2017), cutaneous (Raiesi *et al*. 2017), ocular (Zhao *et al*. 2016) and even systemic infections (Gabrielli *et al*. 2014) may arise, not only in agricultural settings (Porpon *et al*. 2017; Viegas *et al*. 2016), but also in immunocompromised individuals (Wiesmüller *et al*. 2017; Porpon *et al*. 2017), and with increasing resistance (Meis *et al*. 2016) to mainstay antifungal–azole therapy (Park *et al*. 2017).

Importantly, aflatoxins are considered as the most significant mycotoxins due to their occurrence, toxicology and impact on human well-being and crop trade (Gnonlonfin *et al*. 2013). Aflatoxin is mainly a problem in maize because this crop is colonized in the field depending on environmental conditions. Of the other grains, rice is an important dietary source of aflatoxin associated to poor grain-storage in tropical and subtropical areas (Miller

1995). Similarly, fungal aflatoxins have been recently demonstrated to inhibit mucocilliary beating in mammalian airways (Lee *et al*. 2016), thereby contributing to respiratory mucous accumulation and exacerbation of in patients with chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF). Together, the food security and medical risk potential presented by *Aspergillus* spp. and aflatoxins define an unmet need for effective protection and prevention both in crops and man. Yet, to date, traditional pre-harvest control methods, irrigation and identification of plant defence proteins do not appear to be able to control *Aspergillus* crop contamination (Campbell and White 1995; Payne 1998; Chen *et al*. 2010), whereas, the malleability of *Aspergillus* QS and its utility in the agrochemical and respiratory medicine context remains unexplored.

We have recently identified and described the *B. licheniformis* NCIMB 8874 QS pheromone ComX (Esmaeilishirazifard *et al*. 2017). In this study, the pheromone potential of the putative *B. licheniformis* NCIMB 8874 QS genes (*comQX*) was identified through whole genome sequencing and explored through recombinant over-expression in *Escherichia coli* (Esmaeilishirazifard *et al*. 2017). Here, we report for the first time an antifungal property for the *B. licheniformis* ComX pheromone peptide, through antagonism to *A. flavus*. Its impact on fungal growth is analysed using a novel image analysis algorithm (ColonyAreaAnalyzer) developed for this project (Esmaeilishirazifard 2016, supporting information Figs. S1-S3). Furthermore, the impact of this QS peptide and other treatments on fungal growth as well as the biotechnological control of aflatoxin contamination are discussed.

Materials and methods

Strains, media and general methods

B. licheniformis NCIMB 8874, available in the Culture Collection of the University of Westminster, London, UK, was used for investigation of the effect of QSM and bacterial cells on fungal growth. Lysogeny broth (LB) and LB agar (LBA) (Sigma) were used for the maintenance of *B. licheniformis* NCIMB 8874.

Pheromone producer strain (the plasmid was constructed and transferred into *E. coli* BL21 (DE3) by standard techniques) (Esmaeilishirazifard *et al*. 2017) was cultivated in M9 minimal salts solution (sigma). The medium was supplemented with a mixture of filter-sterilised

amino acids (leucine, phenylalanine, histidine, serine, 40 μgml^{-1} each; glutamine, 400 μgml^{-1}), and ampicillin (100 μgml^{-1}). According to the manufacturer instruction, additional supplementation of filter-sterilised 20% (w/v) glucose, 1 M magnesium sulfate and 1 M calcium chloride was required in order to complete M9 minimal medium preparation. Filter sterilisation was carried out through a 0.22 µm filter (Millipore).

A. flavus, NRRL 3357 (aflatoxigenic strain) and ESP 15 (non-aflatoxigenic strain) were kindly obtained from Prof. Naresh Magan, Department of Environmental Science and Technology, Cranfield University. Potato dextrose agar (PDA) was used for propagation and sporulation of *A. flavus* strains. For spore inoculation of the PDA plates, spores were extracted from the fully sporulated slants/plates using sterile 0.01% Tween 80 (v/v) supplemented with 2 mm glass beads (VWR). Spores were counted using a haemocytometer and adjusted to the desired inoculums concentration before inoculating the PDA plates.

Pheromone over-production, purification and fungal-growth test

E. coli BL21 ComX producer strain (Please refer to the method of preparation of this recombinant strain described in our previous work, Esmaeilishirazifard *et al*. 2017) was grown overnight in the completed M9 minimal salts medium described earlier. At stationary phase, this pre-culture (20 ml) was added to 1980 ml of the supplemented M9 medium to make 2 L bacterial culture (5 flasks in total to prepare 10 liters culture) and then incubated at 37 °C and 110 rpm for 8 h. QS gene (*comQX*) expression was induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C and 110 rpm overnight. Before and after the addition of IPTG, 10 ml samples were collected as treatments for the fungal growth test. The culture broth (10 L) was centrifuged for 10 min at 8,000 g. The supernatant was filtered through a 0.22 µm vacuum filtration unit Corning (Sigma). Reverse-phase chromatography method was performed for the initial purification and concentration of the filtered supernatant using Diaion HP-20 resin. The dried extract from reverse-phase chromatography was re-dissolved in 1% DMSO to prepare a solution of 5 and 30 $mgml^{-1}$. To prepared washed cells of *B. licheniformis* NCIMB 8874, the cells were washed three times with sterile water by spinning at 7000 rpm for 10 min. All the treatments and associated controls are listed and labelled in Table S4. The images of the plates were taken every 24 h during the 7-day study. All results are presented as a mean ± standard error of two

independent experiments performed in triplicates. The diagram in Fig. 1 shows the stages that were followed to perform this experiment.

Fungal dry weight measurement

Fungal strains (*A. flavus* NRRL 3357 and ESP 15) grown in the shaken tubes contained 10 ml PDB with all the applied treatments as described for the fungal growth experiment (Table S4). Each tube was inoculated with the prepared spore suspension with the final concentration of 10 spore.ml^{-1}. The cultures were incubated for 7 days in the incubator shaker at 180 rpm, 27 °C. Then they were harvested and filtered using grade No. 54 filter paper (22 µm pore size) (Whatman). These filter papers were kept at 70 °C for 24 h, cooled down to room temperature in desiccators and pre-weighted. Filter papers containing fungal mass were dried at 70 °C in the oven for 48 h, cooled down to room temperature in a desiccator before weighing. The difference between the weight of the filter paper and the combined weight of the filter paper together with fungal dried mycelia was considered as fungal dry weight.

Antifungal susceptibility assays for pheromone

The activities of the purified pheromone determined against *A. flavus* (aflatoxigenic strain) using micro-broth dilution assay (du Toit and Rautenbach 2000). According to the M38-A standard protocol for moulds, the medium RPMI-1640 (Sigma) containing L-glutamine and phenol red (as a pH indicator) (Sigma) was used for growth of *A. flavus* in antifungal susceptibility study. The medium was supplemented with filter-sterilised 0.2% glucose and buffered to a pH of 7.0 with 0.165 molL^{-1} 3-(N-Morpholino) propanesulfonic acid (MOPS) (Sigma) (CLSI M38-A method from CLSI 2008). All procedures were performed under aseptic conditions in sterile 96-wellmicrotiter plates. The fungal spore suspension was prepared using the extracted spores from the fully sporulated plates in 5 ml of sterile 0.01 % Tween 80 (v/v) supplemented with glass beads. Spores were counted using a haemocytometer and adjusted to the desired concentration (2x10^{6} spore.ml^{-1}) to inoculate 5 ml the half strength RPMI-1640 medium. The prepared broth spore suspension (90 µl) was added to the wells (Troskie *et al*. 2012). Each well contained a total of 1.8x10^{4} spores. Dried pheromone mixture was dissolved in 1% Dimethyl sulfoxide (DMSO) to a concentration of 13 µgml^{-1}. Serial dilutions (6.5, 3.25, 1.62, 0.8, 0.4, 0.2, 0.1, 0.05 µgml^{-1}) were made using 1% DMSO

and then 10 µl of these diluted pheromone mixtures were added to the wells containing 90 µl broth suspension. Control culture (positive) received 10 µl of 1% DMSO instead of pheromone. Control negative was a combination of half strength RPMI-1640 (90 µl) and 1% DMSO (10 µl) to confirm the sterile RPMI-1640 and DMSO used in the wells. All wells contained a final volume of 100 µl. Subsequent to the pheromone addition, the microtiter plate was covered tightly with tinfoil, sealed with parafilm and incubated at 27 °C for 48 h. The absorbance of the cultures in the wells was spectrophotometrically determined at 530 nm after 48 h incubation.

Aflatoxin gene expression study in A. flavus using reverse transcriptase polymerase chain reaction (RT-PCR)

A. flavus which was grown on PDA medium with different treatments kept at -20 ºC for this experiment. The cultures were harvested using the scalpel to collect 100 mg fungal biomass. Total RNA was extracted using RNeasy Mini Plant Kit (Qiagen) according to the manufacturer's instructions. All RNA samples were treated by using QIAGEN RNase-Free DNase Set (Qiagen, UK) to digest contaminating genomic DNA in RNA solutions prior to RNA clean up and concentration (using the RNeasy MinElute Cleanup kit (Qiagen, UK)). Complementary DNA (cDNA) synthesis from *A. flavus* mRNA was carried out using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, UK) according to the manufacturer's instructions.

The synthesised cDNA from all RNA samples were amplified using the conventional PCR. The amplification reaction was carried out by utilising PCR master mix (Promega, Southampton, UK). The PCR reaction was prepared according to the manufacture instructions. The PCR conditions were optimised for applying combined primes; *aflp* primers for aflatoxin gene (F-*aflP*/R-*aflP*) and *ITS* primers for housekeeping genes (*ITS1*/*ITS4*) (details are given in Table S5). The amplification program was started with initial denaturation at 94 °C for 2 min and followed with 35 cycles of denaturation at 94 °C, annealing at 55 °C and extension at 72 °C all for 1 min. Final extension was for 5 min at 72 °C. On completion of the PCR reaction, 8 µl of each PCR product was subjected to electrophoresis on a 2% agarose gel containing ethidium bromide. Following electrophoresis, bands corresponding to transcripts of the

study gene, *aflP*, and the reference gene, *ITS*, were noted. The gel was photographed using UVITEC Imaging System (Cambridge, UK).

Results

B. licheniformis NCIMB 8874 and its QSM inhibit the growth of *A. flavus*

To investigate the antifungal potential of *B. licheniformis* NCIMB 8874, we initially examined if this strain had any effects on the growth of *A. flavus* NRL 3375 and the non-aflatoxigenic strain ESP 15. Thus, medium (10^4 cfu/ml) or high (10^9 cfu/ml) concentrations of live *B. licheniformis* cells were mixed with melted potato dextrose agar (PDA), PDA plates were spot inoculated with 10^8 spore.ml^{-1} *A. flavus* and colony growth was documented at daily intervals during a 7-day incubation at 27 ^{o}C (Fig. 1).

These treatments appeared to dose-dependently reduce *A. flavus* growth by ≥ 66% +/- 2.5 in both *A. flavus* species (average inhibition percentage, Fig. 2 and Table S2). To determine whether the observed effect was on account of the ComX pheromone, the *comQX* locus was cloned into *E. coli* BL21 under IPTG control and supernatant filtrates from the resulting *E. coli* transformants were used to prepare *A. flavus* PDA plates. As this resulted only in minor *A. flavus* growth inhibition (7.8% +/- 2.5 in *A. flavus* NRRL 3357; 10.5% +/- 6.0 in *A. flavus* ESP 15) which was nonetheless higher than the no IPTG and *E. coli* BL21 controls, recombinant ComX was purified, concentrated and used instead, at either high (30 mgml^{-1}) or low (5 mgml^{-1}) concentrations. These treatments reduced *A. flavus* growth by 56% +/-8 (NRRL 3357) or 53% +/- 3.5 (ESP 15) at high ComX concentration, and 8% +/-3.5 (NRRL 3357) or 2.5% +/- 1.3 (ESP 15) at low ComX concentration, respectively. As spent, mid-exponential growth phase *B. licheniformis* media had comparable impact on *A. flavus* growth inhibition to the low recombinant ComX preparation, we reasoned ComX levels remained reduced during this stage of *B. licheniformis* growth (Fig. 2 and supplementary information, Tables S1-S3 and Fig. S4).

To examine whether the effect of *B. licheniformis* on *A. flavus* growth was on account of ComX in *B. licheniformis* supernatant, medium (10^4 cfu/ml) or high (10^9 cfu/ml) concentrations of *B. licheniformis* were washed 3x with sterile water and resuspended in sterile water before PDA plate preparation. Interestingly, only the high concentration of washed and resuspended *B. licheniformis* cells impacted upon *A. flavus* growth (48% +/- 5.8

in NRRL 3357 or 41% +/- 1.3 in ESP 15), with the low concentration exhibiting an effect comparable to mid-exponential phase *B. licheniformis* supernatant or ComX-recombinant *E. coli* supernatant. Moreover, the effect of washed *B. licheniformis* cells was 24%-30% lower than that of unwashed cells, suggesting that both cellular and exocytosed ComX contributed to *A. flavus* growth inhibition.

To determine whether these observations translated into a reduced mass of *A. flavus*, these experiments were repeated with suspension *A. flavus* cultures for both *A. flavus* NRRL 3357 and ESP 15, and dry mass was measured after suspension filtration and desiccation (Fig. 3). Thus, dry mass measurements compared to paired control experiments (no treatment) confirmed that the plated *A. flavus* growth inhibition observations were statistically significant ($p < 0.05$, n=3). Additionally, these experiments indicated that recombinant ComX from *E. coli* supernatants was also able to inhibit fungal growth, but that this was statistically significant only in liquid culture. In contrast, the inhibition levels observed in liquid culture were not as relatively extensive as the ones observed on solid PDA media. Interestingly, the findings with aflatoxigenic *A. flavus* NRLL 3357 were reproduced with the non-alfatoxigenic *A. flavus* ESP 15 strain. Taken together, these results raised the hypothesis that *B. licheniformis* ComX may possess broad antifungal properties against *A. flavus*.

To test this hypothesis, the standard, microtitre-based antifungal susceptibility assay CLSI M38-A (CLSI 2008) was carried out to identify a threshold antifungal concentration for the semi-purified ComX pheromone produced in *E. coli* (Fig. 4). Thus, double dilution series starting at 13 $\mu g ml^{-1}$ resulted in 77% growth inhibition ($p \leq 0.05$, n=4) at the highest purified ComX concentration tested. As this is classified as slight growth, score 4, according to the standard CLSI protocol, these data document a high antifungal effect of the pheromone on fungal growth.

B. licheniformis and its ComX QSM inhibit aflatoxin gene expression in *A. flavus*

To explore the impact of these supplementations on aflatoxin production, *A. flavus aflP* gene expression was explored by exon-spanning, end point reverse transcription polymerase chain reaction (RT-PCR), against the *A. flavus ITS* housekeeping gene, as efforts to identify optimally performing, wide range, log-linear real time intercalator primer sets for both genes proved problematic. Thus application of vehicle (DMSO), no treatment control

(water) or supernatants of a washed 10^4 cfu/ml, but not 10^9 cfu/ml *Bacillus* suspension on aflatoxigenic *A. flavus* NRRL 3357 yielded 121 bp cDNA amplicons indicative of *aflP* mRNA production against robust gDNA gene detection (Fig. 5). Furthermore, the data indicated very weak levels of *aflP* expression by this end point, 40 cycle assay, explaining limitations in quantification efforts, and highlighting the inhibitory effect of recombinant ComX in *aflP* expression.

Discussion

Previous studies reported that *Bacillus* species (*B. subtilis* and *B. licheniformis*), *Pseudomonas*, *Ralstonia* and *Burkholderia* strains could completely inhibit *A. flavus* growth and aflatoxin production (Nesci *et al*. 2005; Palumbo *et al*. 2006). Moreover, the bio-control agents *B. subtilis*, *P. fluorescens* and *Trichoderma viride* showed >65% inhibition in *A. flavus* growth (Reddy *et al*. 2010). Our study investigates the antagonistic effect of the *B. licheniformis* strain NCIMB 8874 on aflatoxigenic and non-aflatoxigenic strains of *A. flavus*, and is the first to examine the effects of *B. licheniformis* QSM ComX on fungal growth. Thus, by using different concentrations of *B. licheniformis* and its ComX pheromone recombinantly expressed in *E. coli*, we demonstrate statistically significant growth inhibition of *A. flavus*. These experiments indicate that, over the space of one week, ComX may prevent both the surface spread and reproduction capacity of the fungus, as plate-based growth was inhibited by ~70% and fungus dry mass increase in liquid culture was also significantly curtailed. Furthermore, the relatively weak levels of *aflP* expression, the *A. flavus* gene encoding for aflatoxin, were reduced below assay detection limit after exposure to wild type or recombinant ComX. Exploring such components of organisms antagonistic to *A. flavus* with inhibitory potential to pathogen growth and toxigenicity are expected to provide a better insight into ways to overcome or limit infection in both plants and mammals.

In line with this hypothesis, it has been recently suggested that a *B. licheniformis* strain associated with the marine organism *Spongia officinalis* has inhibitory and anti-biofilm activity on *E. coli* and *P. fluorescens* by secreting a complex exo-polysaccharide compound (Sayem *et al*. 2011). Elsewhere, *A. fumigatus* biofilm formation was inhibited by co-culturing of *P. aeruginosa*; a secreted heat-stable factor (decanol and decanoic acid, analogous to

Pseudomonas QSMs) was shown to exhibit biofilm inhibition. Based on this finding, it was hypothesised that QSMs affected fungal growth (Ramage *et al*. 2011) and could offer a starting point for antifungal drug discovery research efforts. Overall, it has been suggested that small diffusible and heat-stable molecules might be responsible for the competitive inhibition of filamentous fungal growth in environments with potential for polymicrobial colonisation such as the lung, and this could be exploited as a potential therapeutic strategy (Mowat *et al*. 2008; Seidler *et al*. 2008). Indeed, there are other instances in the literature where QSMs have been reported to exhibit antimicrobial activity. Thus, a gram-negative QSM (AHL) and its derived tetramic acid was shown to be bactericidal against Gram-positive bacteria (Kaufmann *et al*. 2005). Tetramic acids have been known to display mycotoxic, antibacterial and antiviral activities (Wang *et al*. 2003; Evans *et al*. 2006; Yu *et al*. 2007).

The current study examined the antagonistic effect of *B. licheniformis* NCIMB-8874 on *Aspergillus* growth under co-culture conditions. The results showed that *B. licheniformis* cells cause a significant reduction in *A. flavus* growth, whether in solid or liquid phase (Fig. 2 and 3). Moreover, at high concentrations of *B. licheniformis* (10^9 cfu/ml inoculum), the effect did not appear to rely entirely on ComX release, as washing of the bacilli did not eliminate their antifungal potential. Accordingly, cell-free supernatant of *B. licheniformis* from the exponential growth phase showed a negligible decrease in the fungal growth, reinforcing the tenet that bacterial cells in the fungal culture are the effective factor on fungal growth reduction rather than the cell-free supernatant. In this scenario, the bacterial cells would compete against fungus, and/or secrete/employ their antimicrobial molecules under the co-culture condition. These results are comparable to previous studies on antagonising microorganisms (Nesci *et al*. 2005; Reddy *et al*. 2010), and indicate *B. licheniformis* may directly antagonise *Aspergillus*.

Alternatively, key nutrient depletion in co-culture conditions and the differential growth rates of *A. flavus* and *B. licheniformis*, especially at the inoculation ratios where 10^9 cfu of *B. licheniformis* were used, could explain these observations. However, the PDA medium is rich enough to make it unlikely that the nutrients would be sequestered by the bacteria, inhibiting *A. flavus* spread to the extent we observed. On the other hand, *B. licheniformis* monoculture may enrich extracellular media with proteins and metabolites distinct to those produced under co-culture with *A. flavus*. In agreement with this interpretation, Losada and

co-workers (Losada *et al*. 2009) performed co-cultivation competition assays among different species of *Aspergillus*, to observe that *Aspergillus* extracts had greater antifungal activity when they were grown in the presence of a competitor. More importantly, gas chromatography experiments supported this hypothesis, indicating that *Aspergillus* extract composition was altered due to the use of competitor organisms. It is thus likely that, although not explicitly tested, these findings extend to the co-culture conditions we have explored herein.

In our hands, both washed and unwashed *Bacillus* cells exerted inhibitory effects on fungal growth, but to different degrees. Thus, assuming ComX as the causal factor to *A. flavus* growth inhibition, washing of *B. licheniformis* cells appeared to reduce antifungal potential, probably by eliminating cell-free ComX from the media. However, as the co-culture assay conditions permitted the washed *B. licheniformis* cells to grow and produce fresh ComX, our data indicated the bacterial cells were able to reinstate ComX to levels inhibitory to *A. flavus* growth. In line with this interpretation, low concentrations of recombinant ComX arising from recombinant *E. coli* supernatants or recombinant *E. coli* cell extracts (5 mg/ml) failed to appreciably inhibit *A. flavus*, as did the supernatants of non-induced recombinant *E. coli* and non-recombinant *E. coli* (Table S2 and S3). Thus, only 30 mg/ml of recombinant ComX *E. coli* extract impacted *A. flavus* growth and spread.

Crucially, the findings with solid *A. flavus* culture were generally well-reproduced with liquid culture assessing the impact of *B. licheniformis* and recombinant ComX in terms of fungal dry weight (Fig. 3). However, in this assay, the supernatants from the recombinant *E. coli* cells induced with IPTG to express ComX resulted in a significant reduction in fungal dry weight. This may be attributable to poorly detectable minor growth reduction in fungal colony surface area, which might not be as easily measurable as loss of dry mass over a 7-day suspension incubation. Thus, the cumulative growth reduction in the dry weight assay over the 7 days incubation period might enable detection of significant change in the whole fungal mass. Alternatively, differential diffusion rates and concentration levels of ComX through solid PDA vs. shaken liquid culture could account for differences in inhibition efficiency across the colony volume.

The fungal inhibition observation showed that dry mass reductions are more notable in solid plated-base culture as opposed to suspension culture. To discuss, specifically, local ComX gradient effects in solid culture would have a more pronounced inhibitory effect to proximal fungal cells, which would result in reduced surface area growth and total mass. Accordingly, the volume occupied by ComX secretions is vastly smaller to that of liquid culture. By stark contrast, rapid diffusion in liquid culture would require much higher absolute quantity of ComX to be produced to achieve concentrations comparable to that in solid culture. Moreover, the substantial differences in microbial physiology between the two growth phases adequately explain our observations. Accordingly, this multifaceted molecular evidence regarding the inhibited processes would be relevant to a drug development program such as using appropriate and condition-relevant models in the future.

Having said that DNA quantification could be an alternative line of evidence for liquid culture which would add another quality control to experiments relevant to the infection control being attempted in a commercial, or nosocomial setting. Indeed, in respiratory care, the concentrations of ComX in airway surface liquid after nebuliser administration would be closer to those achieved in solid culture than bulk volume liquid culture, simply on account of the physiology of the airways.

With the antifungal potential of ComX suggested thus, and the impact of microorganismal growth rates remaining unresolved, we proceeded to reassess the effect of ComX using low nutritional value media (RPMI-1640) in a microtitere plate-based, standard antifungal susceptibility assay. Identifying 13 $\mu g ml^{-1}$ as the minimum inhibitory concentration (MIC), CLSI classification indicated “slight growth” for *Aspergillus* at this ComX pheromone concentration. Crucially, this concentration is comparable to many established antifungal agents (Lewis 2011), even before structure-activity relationship exploration and derivative pharmacology is pursued. This raw potency highlights the pharmacoeconomic potential of pursuing inter-kingdom QS pheromone research.

On the basis of the food safety and medical risks presented by *A. flavus* and related strains through production of aflatoxins, we therefore sought to investigate whether ComX might extend its utility to preventing aflatoxin gene expression. We reasoned this biomarker assay to be a simpler, more sensitive, specific and safe approach than aflatoxin purification and

quantification from ComX-treated *A. flavus* for the early research. However, direct and dose-dependent measurement of aflatoxin production inhibition pharmacology has been induced as part of the future development of the ComX compound.

Leema and colleagues (2011) had successfully used RT-qPCR to investigate the expression of *aflR*, *aflJ*, *aflC* and *aflD* as surrogates of aflatoxin production in *A. flavus* isolated from keratitis patients. Two genes, *aflD* and *aflJ*, encode the enzymes to synthesize sterigmatocystin (aflatoxin precursor), although *aflC* encodes a polyketide synthase and *aflR* is a biosynthesis regulator. All these genes are important in the earlier stages of biosynthesis pathway. As, however, *aflP* encodes O-methyltransferase A, which converts the *A. flavus* aflatoxin B1 precursor sterigmatocystin to O-methylsterigmatocystin, as well as demethylsterigmatocystin to dihydro-O-methylsterigmatocystin (Abdel-Hadi *et al*. 2011), and has been causally linked to aflatoxin production (Rahimi *et al*. 2016), we sought to establish the expression of this nodal enzyme in the aflatoxin biosynthesis pathway instead. Moreover, other members of aflatoxin gene cluster involved in different steps of biosynthetic pathway (e.g. *aflR*, *aflJ*, *aflC* and *aflD*) would be worth to be investigated in order to immensely transcriptomic study on aflatoxin in the future research.

Furthermore, we explored if *B. licheniformis* in general and ComX specifically had a direct effect on the expression levels of this gene. In our hands, testing three separate primer sets for intercalator dye real time RT-PCR failed to identify suitably robust assays, probably due to the very low levels of *aflP* expression as suggested by an endpoint assay (Fig. 5). However, the effect of *bacillus* supernatants and ComX universally reduced *aflP* mRNA levels below the detection limit of this assay. This finding would suggest that QS effect of ComX might extend to aflatoxin production inhibition in addition to antifungal growth properties, ultimately arresting aflatoxin biosynthesis. However, further studies are needed on the expression dynamics of *aflP*. For example, unexpected amplicons in the end point assay may at first glance suggest splicing changes in *aflP* expression, their <75 bp size upon weak gDNA copy amplification is indicative of PCR artefacts. Similarly, problems with quantitative assay performance did not encourage us to explore exhaustively endogenous reference genes beyond *ITS*, as recently elegantly described in *Talaromyces versatilis* (Llanos *et al*. 2015). The report from Llanos and colleague (2015) indicated the reliable reference genes including; *ubcB*, *sac7*, *fis1* and *sarA* genes, as well as *TFC1* and *UBC6* that were

previously validated for their use in *S. cerevisiae*. Unlike these reported reference genes, in the current study, the weak expression of *ITS* (as a reference gene) was obtained from one test, low concentrated washed *Bacillus* cells (Figure 5). The result displays the possible impact of this treatment on the expression level of the conserved gene such as *ITS*. Also, we reasoned that mean geometric average levels of expression of multiple reference genes is relevant only in real time expression normalisation against unexpected treatment effects to so-called housekeeping genes, and for improved target loading normalisation relative to RNA stability. Confirmatory studies on the anti-aflatoxigenicity of ComX or its derivatives will thus require in depth characterisation of their effects on *aflP* both kinetically, and in the context of cell cycle homeostasis (mechanism of action studies).

The effects of ComX on aflatoxin production notwithstanding, it is now well established that QS plays a major role in the inter-kingdom cross-talk. AHL as a bacterial QSM is an example recognised by eukaryotic cells (e.g. animal cells, plants, seaweed and fungi; Dudler and Eberl 2006). Besides, farnesol as a fungal QSM from *C. albicans* has been reported to decrease the production of *Pseudomonas* QSM. It is further suggested that farnesol and the related compounds may participate in interspecies interactions (Cugini *et al*. 2007). Fungi coexist with bacteria in the environment, and bio-chemical exchange between them is a method of communication. The QS-like behaviour in filamentous fungi was reported in *A. terreus* (Schimmel *et al*. 1998), *A. flavus* (Brown *et al*. 2008 and 2009) and *A. nidulans* (Williams *et al*. 2012). It thus seems that QS is an established mechanism used by fungi to modulate mutualresponses to each other and their environment (Sorrentino 2010). Also, instances of small molecule exchange between bacteria and eukaryotes have been reported (Mullard 2009) and examples of QS processes involved in the interspecies communications have emerged (Lu *et al*. 2014). Considering these recent findings, it can be suggested that the communication between *A. flavus* as a filamentous fungus, which has its own QS network, and its *B. licheniformis* antagonist might occur through their QS systems. While this needs to be further investigated, this study reveals the significant influence of *B. licheniformis* NCIMB-8874QSM on growth of *Aspergillus*, expanding evidence suggesting inter-kingdom cross-talk through the QS systems of these organisms.

This study confirmed the antagonistic activity of *B. licheniformis*NCIMB-8874 against *A. flavus*, and the role of the natural antifungal peptide, ComX pheromone, crucial for

bacterial competence. The results support further research into this *B. licheniformis* strain either directly or on its ComX product as a natural antifungal agent to promote a sustainable bio-control strategy for agricultural crops and airways disease, by exploiting inter-kingdom cross-talk either chemically, biochemically, or even through genetic engineering.

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Compliance with Ethical Standards

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Conflict of interest

Author 1 declares that she has no conflict of interest. Author 2 declares that he has no conflict of interest. Author 3 declares that he has no conflict of interest. Author 4 declares that he has no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures legends

Fig. 1 Diagram of the fungal growth experiment

Fig. 2 The *B. licheniformis* ComX pheromone inhibits *A. flavus* growth. The impact of *B. licheniformis* NCIMB 8874 or its ComX pheromone recombinantly expressed in *E. coli* under IPTG control was evaluated on *A. flavus* NRRL 3357 (Panel A) and *A. flavus* ESP 15 (Panel B) cultures at different concentrations and preparation formats. Highly concentrated *B. licheniformis* cells appear to contain and have released ComX to concentrations inhibiting *A. flavus* growth comparable to 30 mg/ml recombinant, ComX-expressing *E. coli* cell extracts. Data representative of two independent experiments in triplicates are presented.

Fig. 3 Fungal dry weight data of *A. flavus* NRLL 3357 (Panel A) and *A. flavus* ESP 15 (Panel B) (Calculated weight, g, of fungal dry mass which was grown in 10 ml PDB in the presence of different treatments, in triplicates). (*) indicates a significant difference between the test and the control ($P \leq 0.05$).

Fig. 4 Antifungal potential of recombinant ComX by CLSI M38-A assay. *A. flavus* NRLL 3357suspension culture was incubated with decreasing concentrations of recombinant ComX in RPMI for 48h and *A. flavus* growth was measured by absorbance at 530 nm. Fungal growth is expressed as the average growth of four replicates +/- standard deviation, relative to the average growth observed among no ComX controls (0 $\mu g ml^{-1}$). Data representative of two independent experiments are presented.

Fig. 5 Endpoint RT-PCR *aflP* expression in *A. flavus* in response to *B. licheniformis* or recombinant ComX treatment. Exon-spanning primers to the *A. flavus aflP* gene yield 181 bp genomic, and 121 bp mRNA-derived amplicons confirmatory of aflatoxigenic *A. flavus* strain identity, and weak *aflP* expression in the absence of the antagonistic *B. licheniformis* ComX pheromone. The white arrow indicates the weak *aflp* mRNA band position in *A. flavus* NRL 3357. A ~600 bp amplicon corresponds to *ITS* amplification is as a reference gene. M: 25 bp marker.