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

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12

13 **Abstract**

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

26 **Key words**

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

29

30

## 1 Introduction

2 Quorum Sensing (QS) is an intra- and interspecies microbial communication process based  
3 on cell-density found in bacteria and fungi (Nealson *et al.* 1970; Fuque *et al.* 1994; Hogan  
4 2006). In many bacterial species, more than one class of compound engage in the network  
5 of regulatory systems controlling the response of a bacterial population to environmental  
6 changes, by regulating diverse genes (Atkinson and Williams 2009). QS mechanisms in  
7 bacteria are often classified into three different cell communication systems operating in i)  
8 Gram-negative bacteria, ii) Gram-positive bacteria, and iii) both groups of bacteria as a  
9 universal QS system (Miller and Bassler 2001). In filamentous fungi, when QSMs of e.g.  
10 *Aspergillus terreus*, *A. flavus* or *A. nidulans* are exogenously added to their own fungal  
11 cultures, they effect morphological changes as well as secondary metabolite production  
12 such as lovastatin, penicillin and aflatoxin (Schimmel *et al.* 1998; Brown *et al.* 2008 and  
13 2009; Williams *et al.* 2012).

14 Inter-kingdom chemical signalling involved in parasitic and symbiotic relationships with  
15 unicellular and complex eukaryotes is mediated through the cross-sensing of pheromone  
16 and pheromone-like compounds, including QSMs (Fox 2004; Rumbaugh 2007; Mullard  
17 2009). These communication networks are also subject to significant plasticity on account of  
18 enzymatic modification and degradation of the signalling molecules involved. For instance,  
19 one of the bacterial QSMs, Acyl-Homoserine Lactone (AHL), which directs bacterial  
20 compound synthesis, is also recognised by animal, plant, seaweed and fungal cells (Dudler  
21 and Eberl 2006). Animal cell gene expression in response to QSMs has been reported to be  
22 altered in an immune modulatory fashion (Sperandio 2003; Shiner *et al.* 2005). In green  
23 seaweed, Joint *et al.* (2002) demonstrated that *Enteromorpha* zoospores are attracted by  
24 synthetic AHLs to settle on bacterial biofilm microcolonies in an AHL concentration-  
25 dependent manner. In higher plants, the *Medicago truncatula* legume (Mathesius *et al.*  
26 2003) and the tomato rhizosphere (Schuhegger *et al.* 2006) have also been reported to  
27 respond to bacterial AHL signalling. In the legumes, the concentration of more than 150  
28 proteins is altered, whereas AHL in tomato rhizospheres increases salicylic acid  
29 concentration in the plants' leaves, thereby enhancing systemic resistance against the  
30 fungal leaf pathogen *Alternaria alternata*. The latter evidence suggests that AHLs play an

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

3 On the other hand, several reports indicate that fungi interfere with bacterial QS by  
4 producing AHL antagonists (Rasmussen *et al.* 2005). Plant defence signals such as salicylic  
5 acid have been reported to up-regulate the expression of an AHL-degrading enzyme in  
6 *Agrobacterium tumefaciens*. Alternatively, AHL-antagonists are produced by plants and  
7 fungi to block bacterial QS (Bauer and Mathesius 2004; Dudler and Eberl 2006; Williams  
8 2007). Elsewhere, it has been reported that the *Candida albicans* QSM compound, farnesol,  
9 suppresses transcription of the *Pseudomonas aeruginosa* quinolone signal (PQS) QSM  
10 (Cugini *et al.* 2007). Interestingly, Lee and colleagues (Lee *et al.* 2015) recently reported that  
11 farnesol can also block eukaryotic signalling pathways (e.g. STAT3) in mice, potentially  
12 exerting, anti-proliferation and pro-apoptotic activities on tumours.

13 Inter-kingdom communication also underpins food security through regulation of the  
14 metabolism of fungi with pathogenic and saprophytic potential in agricultural crops.  
15 *Aspergillus* species are common soil saprophytes found worldwide, responsible for pre-  
16 harvest and post-harvest disease in several agricultural seed crops (Amaiike and Keller 2011).  
17 Among these fungi, *A. flavus* and *A. parasiticus* are the predominant species responsible for  
18 aflatoxin contamination of crops prior to harvest or during storage (Miller 1995; Yu *et al.*  
19 2004; Gokmen *et al.* 2005). At the same time, *A. fumigatus* and *A. flavus* are the primary  
20 causes of mould aspergillosis in man. Thus, respiratory (Dufresne *et al.* 2017), cutaneous  
21 (Raiesi *et al.* 2017), ocular (Zhao *et al.* 2016) and even systemic infections (Gabielli *et al.*  
22 2014) may arise, not only in agricultural settings (Porpon *et al.* 2017; Viegas *et al.* 2016), but  
23 also in immunocompromised individuals (Wiesmüller *et al.* 2017; Porpon *et al.* 2017), and  
24 with increasing resistance (Meis *et al.* 2016) to mainstay antifungal–azole therapy (Park *et al.*  
25 *et al.* 2017).

26 Importantly, aflatoxins are considered as the most significant mycotoxins due to their  
27 occurrence, toxicology and impact on human well-being and crop trade (Gnonlonfin *et al.*  
28 2013). Aflatoxin is mainly a problem in maize because this crop is colonized in the field  
29 depending on environmental conditions. Of the other grains, rice is an important dietary  
30 source of aflatoxin associated to poor grain-storage in tropical and subtropical areas (Miller

1 1995). Similarly, fungal aflatoxins have been recently demonstrated to inhibit mucocilliary  
2 beating in mammalian airways (Lee *et al.* 2016), thereby contributing to respiratory mucous  
3 accumulation and exacerbation of in patients with chronic obstructive pulmonary disease  
4 (COPD) or cystic fibrosis (CF). Together, the food security and medical risk potential  
5 presented by *Aspergillus* spp. and aflatoxins define an unmet need for effective protection  
6 and prevention both in crops and man. Yet, to date, traditional pre-harvest control  
7 methods, irrigation and identification of plant defence proteins do not appear to be able to  
8 control *Aspergillus* crop contamination (Campbell and White 1995; Payne 1998; Chen *et al.*  
9 2010), whereas, the malleability of *Aspergillus* QS and its utility in the agrochemical and  
10 respiratory medicine context remains unexplored.

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

## 21 **Materials and methods**

### 22 ***Strains, media and general methods***

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

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

1 amino acids (leucine, phenylalanine, histidine, serine, 40  $\mu\text{gml}^{-1}$  each; glutamine,  
2 400  $\mu\text{gml}^{-1}$ ), and ampicillin (100  $\mu\text{gml}^{-1}$ ). According to the manufacturer instruction,  
3 additional supplementation of filter-sterilised 20% (w/v) glucose, 1 M magnesium sulfate  
4 and 1 M calcium chloride was required in order to complete M9 minimal medium  
5 preparation. Filter sterilisation was carried out through a 0.22  $\mu\text{m}$  filter (Millipore).

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

### 13 ***Pheromone over-production, purification and fungal-growth test***

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

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

### 3 ***Fungal dry weight measurement***

4 Fungal strains (*A. flavus* NRRL 3357 and ESP 15) grown in the shaken tubes contained  
5 10 ml PDB with all the applied treatments as described for the fungal growth experiment  
6 (Table S4). Each tube was inoculated with the prepared spore suspension with the final  
7 concentration of 10 spore.ml<sup>-1</sup>. The cultures were incubated for 7 days in the incubator  
8 shaker at 180 rpm, 27 °C. Then they were harvested and filtered using grade No. 54 filter  
9 paper (22 µm pore size) (Whatman). These filter papers were kept at 70 °C for 24 h, cooled  
10 down to room temperature in desiccators and pre-weighed. Filter papers containing fungal  
11 mass were dried at 70 °C in the oven for 48 h, cooled down to room temperature in a  
12 desiccator before weighing. The difference between the weight of the filter paper and the  
13 combined weight of the filter paper together with fungal dried mycelia was considered as  
14 fungal dry weight.

### 15 ***Antifungal susceptibility assays for pheromone***

16 The activities of the purified pheromone determined against *A. flavus* (aflatoxigenic strain)  
17 using micro-broth dilution assay (du Toit and Rautenbach 2000). According to the M38-A  
18 standard protocol for moulds, the medium RPMI-1640 (Sigma) containing L-glutamine and  
19 phenol red (as a pH indicator) (Sigma) was used for growth of *A. flavus* in antifungal  
20 susceptibility study. The medium was supplemented with filter-sterilised 0.2% glucose and  
21 buffered to a pH of 7.0 with 0.165 molL<sup>-1</sup> 3-(N-Morpholino) propanesulfonic acid (MOPS)  
22 (Sigma) (CLSI M38-A method from CLSI 2008). All procedures were performed under aseptic  
23 conditions in sterile 96-well microtiter plates. The fungal spore suspension was prepared  
24 using the extracted spores from the fully sporulated plates in 5 ml of sterile 0.01 % Tween  
25 80 (v/v) supplemented with glass beads. Spores were counted using a haemocytometer and  
26 adjusted to the desired concentration (2x10<sup>6</sup> spore.ml<sup>-1</sup>) to inoculate 5 ml the half strength  
27 RPMI-1640 medium. The prepared broth spore suspension (90 µl) was added to the wells  
28 (Troskie *et al.* 2012). Each well contained a total of 1.8x10<sup>4</sup> spores. Dried pheromone  
29 mixture was dissolved in 1% Dimethyl sulfoxide (DMSO) to a concentration of 13 µgml<sup>-1</sup>.  
30 Serial dilutions (6.5, 3.25, 1.62, 0.8, 0.4, 0.2, 0.1, 0.05 µgml<sup>-1</sup>) were made using 1% DMSO

1 and then 10 µl of these diluted pheromone mixtures were added to the wells containing  
2 90 µl broth suspension. Control culture (positive) received 10 µl of 1% DMSO instead of  
3 pheromone. Control negative was a combination of half strength RPMI-1640 (90 µl) and 1%  
4 DMSO (10 µl) to confirm the sterile RPMI-1640 and DMSO used in the wells. All wells  
5 contained a final volume of 100 µl. Subsequent to the pheromone addition, the microtiter  
6 plate was covered tightly with tinfoil, sealed with parafilm and incubated at 27 °C for 48 h.  
7 The absorbance of the cultures in the wells was spectrophotometrically determined at  
8 530 nm after 48 h incubation.

### 9 ***Aflatoxin gene expression study in A. flavus using reverse transcriptase polymerase chain*** 10 ***reaction (RT-PCR)***

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

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

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

### 3 **Results**

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

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

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

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

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

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

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

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

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

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

## 7 **Discussion**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

11 The effects of ComX on aflatoxin production notwithstanding, it is now well established that  
12 QS plays a major role in the inter-kingdom cross-talk. AHL as a bacterial QSM is an example  
13 recognised by eukaryotic cells (e.g. animal cells, plants, seaweed and fungi; Dudler and Eberl  
14 2006). Besides, farnesol as a fungal QSM from *C. albicans* has been reported to decrease the  
15 production of *Pseudomonas* QSM. It is further suggested that farnesol and the related  
16 compounds may participate in interspecies interactions (Cugini *et al.* 2007). Fungi coexist  
17 with bacteria in the environment, and bio-chemical exchange between them is a method of  
18 communication. The QS-like behaviour in filamentous fungi was reported in  
19 *A. terreus* (Schimmel *et al.* 1998), *A. flavus* (Brown *et al.* 2008 and 2009) and *A. nidulans*  
20 (Williams *et al.* 2012). It thus seems that QS is an established mechanism used by fungi to  
21 modulate mutualresponses to each other and their environment (Sorrentino 2010). Also,  
22 instances of small molecule exchange between bacteria and eukaryotes have been reported  
23 (Mullard 2009) and examples of QS processes involved in the interspecies communications  
24 have emerged (Lu *et al.* 2014). Considering these recent findings, it can be suggested that  
25 the communication between *A. flavus* as a filamentous fungus, which has its own QS  
26 network, and its *B. licheniformis* antagonist might occur through their QS systems. While  
27 this needs to be further investigated, this study reveals the significant influence of  
28 *B. licheniformis* NCIMB-8874QSM on growth of *Aspergillus*, expanding evidence suggesting  
29 inter-kingdom cross-talk through the QS systems of these organisms.

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

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

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## 8 **Compliance with Ethical Standards**

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## 12 **Conflict of interest**

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

## 16 **Ethical approval**

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

19

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1 **Figures legends**

2 **Fig. 1** Diagram of the fungal growth experiment

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

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

14 **Fig. 4** Antifungal potential of recombinant ComX by CLSI M38-A assay. *A. flavus* NRLL  
15 3357 suspension culture was incubated with decreasing concentrations of recombinant  
16 ComX in RPMI for 48h and *A. flavus* growth was measured by absorbance at 530 nm. Fungal  
17 growth is expressed as the average growth of four replicates +/- standard deviation, relative  
18 to the average growth observed among no ComX controls ( $0 \mu\text{gml}^{-1}$ ). Data representative of  
19 two independent experiments are presented.

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

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