Composition and activity of antifungal lipopeptides produced by *Bacillus* spp. in *daqu* fermentation

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Abstract

Daqu is a solid-state fermentation and saccharification starter for the Chinese liquor *baijou*. During the *daqu* stage, amylolytic and proteolytic enzymes are produced by *Bacillus* and fungi. *Bacillus* spp. also produce lipopeptides with a broad spectrum of antimicrobial activities but direct evidence for their impact on community assembly in *daqu* is lacking. This study aimed to study the interaction between *Bacillus* spp. and fungi in *daqu* models. The antifungal activity of surfactin, fengycin, and iturin A was initially assessed *in vitro*. Iturin A displayed the strongest antifungal activity (MIC=10-50 mg/L). *In situ* antifungal activity of *B. amyloliquefaciens* and *B. velezensis* against molds was observed in a simple *daqu* model inoculated with single strains of *Bacillus* species. Formation of lipopeptides *in situ* was supported by quantification of mRNA encoding for enzymes for surfactin, fengycin, and iturin A biosynthesis. *In situ* antifungal activity of *Bacillus* species was also observed in a complex *daqu* model that was inoculated with 8 bacterial or fungal strains plus one of the three strains of *Bacillus*. A relationship of lipopeptides to *in situ* antifungal activity was supported by detection of the lipopeptides by liquid chromatography coupled to mass spectrometry. Both results indicated that *B velezensis* FUA2155 had higher antifungal activity in the *daqu* model, and was the only strain that produced multiple iturin A congeners *in situ*. Taken together, this study provides evidence that production of lipopeptides by *Bacillus* species in *daqu* may impact community assembly and hence product quality.

Keywords: *Bacillus*; *daqu* fermentation; antifungal lipopeptides; mass spectrometry.

1 Introduction

Chinese liquor (*baijiu*) is a distilled liquor and one of the most popular alcoholic beverages in China (Zheng and Han, 2016). Chinese liquor is made with sorghum, wheat, rice, barley, or corn (Zheng et al., 2011; Zheng and Han, 2016). Differing from alcoholic cereal fermentations in Africa and Europe, where malt is used as a source of enzymes, hydrolytic enzymes are provided by microbial saccharification cultures including *daqu*, which is produced by spontaneous fermentation of cereals (Gänzle, 2022; Jin et al., 2017; Marco et al., 2021). *Baijiu* also differs from Japanese sake, which uses back-slopped *koji* with domesticated strains of *Aspergillus oryzae* as main or sole fermentation organism (Gibbons et al., 2012). The fermentation process of Chinese liquor consists of two stages: production of the saccharification starter *daqu* and the mash fermentation for ethanol production (He et al., 2019; Huang et al., 2017). *Daqu* has been divided into three categories: low-, medium-, and high-temperature *daqu*, which are used to produce Chinese liquor with light flavor, soy sauce flavor and strong flavor, respectively (Sakandar et al., 2020). The medium-temperature *daqu* is the most widely used starter in the production of traditional Chinese *baijiu* (Xiao et al., 2017).

The composition of microorganisms in *daqu* consists of bacteria, mycelial fungi and yeasts (Chen et al., 2021). Bacterial species include *Bacillus* spp., *Enterobacteriaceae* and *Lactobacillaceae*. *Bacillus* species are endophytes of plants and thus invariably present in the raw materials of *daqu* preparation (Ferreira et al., 2021; Leite et al., 2013), and are consistently found to be the dominant species throughout the fermentation (Chen et al., 2021; Wang et al., 2008; Zheng et al., 2013). Fungal strains consist of *Aspergillus* spp., *Mucor* spp., *Penicillium* spp., with *Aspergillus* spp. being dominant (Deng et al., 2021; Wang et al., 2008). *Saccharomyces* spp. are most frequently isolated yeasts (Wang et al., 2008).

Daqu is produced using unsterilized raw materials in an open environment without starter culture or inoculum and fermentation organisms are derived from the raw material or the environment. *Bacillus* spp. form stable associations with plants in the rhizosphere or as endophytes (Robinson et al., 2016; Shahzad et al., 2016), and thus are invariably present in cereal grains (Li et al., 2020). The shape of the *daqu* blocks provides a large surface area to support the growth of the aerobic bacilli, and the low moisture content slows the growth of *Enterobacteriaceae* and lactic acid bacteria (Zheng and Han, 2016). These ecological parameters thus allow *Bacillus* spp. to dominate throughout the production process (Zheng et al., 2013). Endospores formed by *Bacillus* remain active at low moisture content and high-temperature conditions (Setlow, 2006), supporting their presence as the most frequently isolated bacteria from *daqu*.

During the *daqu* production, hydrolytic enzymes including amylolytic enzymes are produced by bacteria and fungi (Li et al., 2014; Liu et al., 2018). Of the bacterial fermentation organisms, *Bacillus* spp. are the major group producing extracellular amylolytic enzymes (Li et al., 2014; Liu et al., 2018). The amylolytic system have been identified in genomes of *Bacillus* spp. (Li et al., 2020). Amylases produced in *daqu* are the major contributors to starch liquefaction and saccharification in the subsequent mash fermentation (Li et al., 2015). In addition, proteolytic enzymes produced by fungi and bacilli generate amino acids as precursors for synthesis of volatile flavor compounds in the mash stage (Liu et al., 2018).

Bacillus spp. also produce a wide range of lipopeptides with antimicrobial activity (Cochrane and Vederas, 2016; Zhang et al., 2022). These lipopeptides are produced by polyketide synthases (PKSs) and nonribosomal peptide-synthetases (NRPS) (Roongsawang et al., 2010). Lipopeptides produced by *Bacillus* spp. can be broadly grouped into three families: surfactins, fengycins and iturins (Cochrane and Vederas, 2016). These antifungal lipopeptides inhibit or kill fungi either by

inhibiting mycelial growth, affecting spore germination, or causing the hyphae or spores to swell or to lyse (Li et al., 2021). Genes coding for synthesis of antimicrobial lipopeptides including *bioA*, *bmyB*, *ituC*, *fenD*, *srfAA*, *srfAB*, *yngG*, and *yndJ*, were identified in the genomes of bacilli isolated from *daqu* (Wu et al., 2021). In addition, *Bacillus* lipopeptides were identified in both *daqu* and *baijiu* (Chen et al., 2020; Zhang et al., 2014). For example, surfactin accumulated to a concentration of more than 7 mg/kg in the *daqu* stage of *baijou* fermentation and was diluted to 1.5 mg/kg by the addition of cooked cereals at the mash stage. The concentration of the non-volatile peptide in the distilled end product was less than 1 µg/L (Chen et al., 2020). It remains unknown, however, whether the production of antifungal lipopeptides in *daqu* impact community assembly in *daqu* and mash fermentations. It was therefore the aim of this study to investigate the role of antifungal lipopeptides produced by *Bacillus* species on community assembly in *daqu*.

2 Materials and methods

2.1 Strains used in this study and preparation of the inocula

The origin and growth conditions of strains used in this study are listed in Table 1. LB broth (SigmaAldrich, ON, Canada) was inoculated with a single colony of *Bacillus amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82, *Bacillus velezensis* FUA2155, or *Kosakonia cowanii* FUA10121 and incubated overnight at 37 °C at 200 rpm agitation. *Weissella cibaria* FUA3456 cultures was prepared in a similar manner but the strain was grown in modified MRS (mMRS) broth (Gänzle et al., 1998) at 30 °C for 2 d without agitation.

Spore suspensions of the following fungal strains, *Aspergillus niger* FUA5001, *Aspergillus clavatus* FUA5004, *A. clavatus* FUA5005, *Mucor racemosus* FUA5009, and *Penicillium roqueforti* FUA5012 were prepared as described (Zhang et al., 2010). In brief, the strains were cultivated on malt extract agar (MEA, SigmaAldrich, ON, Canada) plates at 25 °C for

7 d. Conidia were collected from agar plates by adding 10 mL of sterile distilled water and harvesting of fungal biomass with an L-shaped cell spreader (Fisher Scientific, Ottawa, Canada). The spore suspensions were filtered to eliminate mycelial cells and spores were harvested by centrifugation. Spores were quantified with a haemocytometer (Fein-Optik, Jena, Germany).

Inocula of *Saccharomyces cerevisiae* FUA4002, *Saccharomycopsis fibuligera* FUA4036, and *Pichia kudriavzevii* FUA4039 were prepared by inoculating malt extract broth (MEB, SigmaAldrich, ON, Canada) with single colonies, followed by incubation for 2 d at 30 °C. The cell counts were confirmed with a hemocytometer.

2.2 *In silico* prediction of lipopeptides produced by *Bacillus* spp.

The genome sequences of *Bacillus* strains (Li et al., 2019) were used to identify biosynthetic gene clusters for antimicrobial secondary metabolites using the bacterial version of the antiSMASH (Blin et al., 2021). Identification of gene clusters encoding different lipopeptides in the genomes of *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82 and *B. velezensis* FUA2155 were verified by BLAST on NCBI. All gene clusters identified by antiSMASH were used as query sequences for BLASTn against the NCBI nucleotide database.

2.3 Extraction and purification of the antifungal peptides from LB cultures

To study the antifungal peptides produced by *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82 and *B. velezensis* FUA2155, the peptides were extracted from 150 mL of the stationary phase cultures in LB broth. The cultures were incubated at 37 ℃, 200 rpm for 3 d and cells were removed by centrifugation at 12,000 × *g* for 20 min. The pH of the supernatants was adjusted to 2 with 6M HCl, followed by incubation at 4 ℃ overnight. Solid crude lipopeptides were collected by centrifugation at 12,000 × *g* for 20 min, and the precipitate was extracted with methanol. The

organic solvent was evaporated *in vacuo* at 50 ℃ (Yang et al., 2015). The extracted peptides were dissolved in 1 mL methanol and filtered through 0.45 μm filters to remove solids.

2.4 Minimum inhibitory concentration assay

The minimum inhibitory concentration (MIC) of surfactin, fengycin and iturin A was determined in a critical dilution assay. Iturin A and fengycin (Sigma-Aldrich, Oakville, Canada) and surfactin (MedChemExpress, Monmouth Junction, USA) were dissolved in DMSO to a concentration of 5 g/L as stock solutions and stored at –80 ℃ until further use. The mycelial fungi *A. niger* FUA5001, *A. clavatus* FUA5004, *A. clavatus* FUA5005, *M. racemosus* FUA5009 and *P. roqueforti* FUA5012, as well as the yeasts, *S. cerevisiae* FUA4002, *S. fibuligera* FUA4036 and *P. kudriavzevii* FUA4039 were used as indicator strains. Inocula were prepared as described above. The growth was observed visually, and the MIC was recorded one day after visible growth. The MIC values were determined in three independent experiments using replicate preparations of the conidiospores. For each experiment, 90 μL of the lipopeptides iturin, surfactin, fengycin were mixed with MEB (for fungi) or mMRS broth (for yeasts) in a 96-well microtiter plate, followed by 2-fold serial dilutions with the respective growth medium to cover the concentration range of 2.5 g / L to 2.5 mg / L. Each well was inoculated with 10 μL of spores or vegetative cells with a cell count of 10^6 CFU/mL. The controls contained the inocula but distilled water was used instead of the lipopeptide solutions.

2.5 Preparation of simplified *daqu* model

The production of antifungal peptides by the three strains of *Bacillus* (*B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82 and *B. velezensis* FUA2155) was initially assessed in a simplified *daqu* model that were inoculated with only one *Bacillus* strain. Overnight cultures of the three *Bacillus* strains were prepared by inoculating single colonies in 100 mL LB broth that

was incubated at 37 °C and 200 rpm for 24 h. Cells were harvested by centrifugation and resuspended in sterile water to obtain a cell count of 10^9-10^{10} CFU/mL. One mL of this inoculum, 60 g wheat flour and 30 mL sterile tap water were mixed in a sterile plastic bag, resulting in a water content of around 35 % and a final cell count of approximately 10^7-10^8 CFU/g. Wheat flour mixed with sterile water without inoculation of strain of *Bacillus* served as the control. The *daqu* samples were manually pressed and shaped in Petri-dishes and incubated at controlled temperatures and relative humidity (rH) as follows: shaping stage, 30 °C, rH 95 % for 1 d; ripening stage, 37 °C, rH 95 % for 2 d; high-temperature stage, 55 °C, rH 90 % for 7 d; and maturation stage, 37 °C, rH 75 % for 6 d. This temperature and relative humidity profile matches conditions of medium-temperature *daqu* conditions (Zheng and Han, 2016). Samples were incubated in hermetically sealed containers and the relative humidity in these containers was controlled with the following saturated salt solutions: K_2SO_4, rH 95 %; KNO_3, rH 90 %; NaCl, rH 75 %. After the high-temperature stage, the *daqu* samples were dry and microbial population remained stable, the incubation was therefore not extended for more than a total of 16 d.

2.6 Determination of pH and total bacterial cell counts of the simplified *daqu* model, and observation of mold growth

To measure the pH and viable cell counts, 0.5 g samples were collected and diluted 10-fold with sterile 18 MΩ water. Samples were further diluted in peptone water and plated on LB agar. The pH of the first dilution was then measured with a glass electrode. The LB agar plates were incubated at 37 °C for 24 h prior to counting the total number of colonies per plate; differential cell counts of *Bacillus* species were also recorded based on the colony morphology.

Fungal growth during the simplified *daqu* model was observed daily and recorded as follows: –, no mycelial growth visible; +, small spots of mycelial growth; ++, spots of mycelial growth and

conidia; +++, 25-50 % of the surface covered by mycelium; and ++++, more than 50 % pf the surface covered by mycelium. Representative pictures defining the fungal growth are shown in the Supplementary Figure S1.

2.7 Quantification of expression of genes encoding for synthesis of three antifungal lipopeptides in the simplified *daqu* model by reverse transcription quantitative PCR (RT-qPCR)

To extract mRNA and prepare cDNA of the 1st, 2nd, and 3rd day of samples from the the simplified *daqu* model, aliquots of 0.5 g *daqu* were mixed with 3 mL RNAprotect Bacteria Reagent (Qiagen, Germantown, USA) and incubated for 10 min. The solids were then removed by centrifugation at 500 × *g* for 10 min. The cells in the supernatant were harvested by centrifugation. The RNA was isolated from cell pellets using TRIzol LS reagent based on the manufacturer's instructions (ThermoFisher Scientific, Waltham, USA). Contaminant genomic DNA was digested by DNase treatment using the RQ1 RNase-Free DNase (Fisher Scientific), and cDNA libraries were generated by reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen).

To quantify the expression of genes encoding surfactin, fengycin and iturin A production in the *daqu* model, the genes *srfAA, fenA and ituA* encoding part of the nonribosomal peptide synthetase subunits of surfactin, fengycin and iturin A, respectively, were chosen. Primers used in qPCR are shown in Table 2 and the specificity of the primers was verified by PCR using chromosomal DNA of the three strains as a template. The single-copy gene *gyrB* which encodes for the DNA gyrase subunit B was used as the housekeeping gene for relative quantification of gene expression.

Gene expression was detected by QuantiFast SYBR Green PCR Kit (Qiagen) and reverse-transcriptase qPCR (7500 Fast, Applied Biosystems, Foster City, CA). Cells growing exponentially in LB broth (OD_{600nm} 0.8) were used as reference conditions. Negative controls

included DNase-treated RNA and a no-template control. Gene expression relative to cultures in LB was calculated with the $\Delta\Delta C_T$ method and $\log_2$ transformed. Significant differences in the relative gene expression were assessed with a t-test and an error probability of 5 % ($P < 0.05$). Data are presented as mean ± standard deviation of three independent fermentations.

2.8 Preparation of complex model of *daqu* fermentation

To further study the role of antifungal peptides produced by *Bacillus* spp. in *daqu*, a complex *daqu* model was prepared by including one representative of major groups of fermentation organisms. In addition to inoculation with strains of *Bacillus*, cell or spore suspensions of *A. niger* FUA5001, *M. racemosus* FUA5009, and *P. roqueforti* FUA5012, *S. cerevisiae* FUA4002, *S. fibuligera* FUA4036, and *P. kudriavzevii* FUA4039 were added to a final cell count of 10^5 CFU/g each. Cell suspensions of *K. cowanii* FUA10121 and *W. cibaria* FUA3456 were added to a final cell count of 10^6 CFU/g each. The samples from the complex *daqu* model were prepared and incubated at the same conditions with the simplified *daqu* model as described above.

2.9 Determination of microbial population in the complex model of *daqu* by qPCR

Samples from the complex *daqu* model were collected after 1, 3, 6, 10, 13 or 16 days of fermentation were collected. Community DNA was extracted by E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Inc. Norcross, USA) according to the manufacturer's instructions. The absolute quantities of microbial populations were determined with qPCR using primers targeting *Bacillus* species, all bacteria, and fungi with the primer pairs *srfAA*F and *srfAA*R, 340F and 758R (Juck et al., 2000), and Fnpstr and Rnpstr, respectively (Rodríguez et al., 2012). The assays were carried out on a 7500 Fast instrument (Applied Biosystems) with a commercial QuantiFast SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. The calibration curves and amplification conditions were constructed as described elsewhere (Metzler-Zebeli et al., 2010). In short, the PCR

amplicons was amplified with chromosomal DNA from *B. velezensis* or *A. niger* FUA5001, and purified. The DNA concentration of the amplicons was measured with a Nanodrop UV/Vis spectrophotometer (Thermofisher) to calculate the copy number, and the DNA solutions were diluted in serial tenfold dilutions to exhaustion. The diluted DNA solutions were used as template for qPCR to establish the calibration curves.

2.10 Extraction and purification of lipopeptides from the complex *daqu* model

To monitor the production of antifungal lipopeptides in the complex *daqu* model, samples were obtained on the 1st, 3rd, 6th 10th, 13th, and 16th day of incubation. The extraction procedure for lipopeptides was similar to that described above for extraction from LB cultures but with an additional homogenization step. Approximately 8.5 g of the *daqu* samples were homogenized in 50 mL distilled water by stomaching for 5 minutes. The samples were then centrifuged at 12,000 × *g* for 20 min to remove solids. Samples were further processed with same procedure as described above for cultures in LB.

2.11 Antifungal activity test of peptides produced by *Bacillus* in the complex *daqu* model

To determine the production of antifungal peptides in the complex *daqu* model, the antifungal activity of methanolic *daqu* extracts was determined with *A. niger* FUA5001 as the indicator strain. Serial 2-fold dilutions of the peptide extracts and MEB were prepared in 96-well microtiter plates and inoculated with 10 μL of a suspension of conidia *A. niger* FUA5001 to a cell count of 10^6 CFU/mL. The plates were then incubated at 25 ℃ for 5 d. After incubation, fungal growth was observed visually.

2.12 Procedure for LCMS analysis of LB culture and complex model of *daqu* extracts

To qualitatively identify the antifungal lipopeptides produced by *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82, and *B. velezensis* FUA2155, each sample was detected first by reverse phase-high performance liquid chromatography followed by coupled to using mass spectrometry (RP-HPLC-MS). Analysis was performed using an Agilent 1200 SL HPLC System with a Phenomenex Aeris XB-C8 column, 3.6 µm, 100 Å, 50 x 2.1 mm with a trap cartridge (Phenomenex, Torrance, USA) with a guard thermostated at 35 °C. A buffer gradient composed of 0.1 % formic acid in water (mobile phase A) and 0.1 % formic acid in acetonitrile (mobile phase B) was used. A 2 µL aliquot of the sample was loaded onto the column at a flow rate of 0.45 mL/min and an initial buffer composition of 95 % mobile phase A and 5 % mobile phase B for 0.5 min was performed to effectively remove the salts. The elution of the lipopeptides was performed using a linear gradient from 5 % to 65 % mobile phase B for 4.8 min, 65 % to 95 % mobile phase B for 1.0 min, 95 % mobile phase B for 0.8 min, then back to the initial buffer conditions in 0.5 min. Mass spectra were acquired in a positive mode of ionization using an Agilent 6220 Accurate-Mass TOF HPLC/MS system (Santa Clara, CA, USA), equipped with a dual sprayer electrospray ionization source; the second sprayer providing a reference mass solution. Mass correction was performed for each individual spectrum using peaks at *m/z* 121.0509 and 922.0098 from the reference solution. The conditions for mass spectrometry are as stated: drying gas 10 L/min at 350 °C, nebulizer at 30 psi, mass range of 100-3200 Da, acquisition rate of ~1.03 spectra/sec, fragmentor voltage at 175 V, skimmer voltage at 65 V, capillary voltage at 4000 V, and instrument state 2 GHz High Dynamic Range. Data analysis was performed using the Agilent MassHunter Qualitative Analysis software package version B.07.00 SP2.

3 Results

3.1 Prediction of antimicrobial lipopeptides produced by *B. amyloliquefaciens* and *B. velezensis*

The production of antimicrobial peptides in the genomes of *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82 and *B. velezensis* FUA2155 was initially predicted by antiSMASH (Supplementary Table S1). All three strains were predicted to produce several antimicrobial lipopeptides. The predicted number and percent sequence identity of peptides in the two strains of *B. amyloliquefaciens* were identical. *B. velezensis* FUA2155 was predicted to additionally produce three antimicrobial peptides including a second surfactin gene cluster. The genome of *B. velezensis* FUA2155 also included gene clusters encoding for the synthesis of the antibacterial peptides difficidin and macrolactin H with high identity. Difficidin has both antifungal and antibacterial activities (Im et al., 2020), while macrolactins have broad-spectrum antimicrobial activity (Yuan et al., 2016). Macrolactin H exhibited antibacterial activity (MIC = 10 mg/L) against *Staphylococcus aureus* (Nagao et al., 2001) but antifungal activity has not been described.

Predictions of the antifungal peptides by antiSMASH were verified by BLASTn (Supplementary Table S2). No significant similarity was found for butirosin A/B and the query coverage for bacillibactin, bacilysin and fengycin biosynthetic genes in the genomes of the three *Bacillus* strains were below 60 %. The query coverage and identity of bacillaene, surfactin and iturin in the three strains were above 60 % (Supplementary Table S2). Based on the presence of gene clusters for antimicrobial lipopeptides, and literature data on their antifungal activity, the peptides surfactin, iturin and fengycin were selected for subsequent experiments.

3.2 Analysis of antifungal lipopeptides in the LB cultures of *Bacillus*

To determine whether the presence of gene clusters coding for production of three families of antifungal lipopeptides surfactin, fengycin and iturins lipopeptides result in production of these lipopeptides during growth in LB broth, total lipopeptides were extracted from LB culture supernatants of *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82 and *B. velezensis* FUA2155. Signal intensities of surfactin, iturin A, and fengycin were qualitatively analyzed by HPLC-MS, and the log[signal intensities] are shown in a gradient (Figure 1). Of the three families of lipopeptide tested, surfactins were detected in all three strains of *Bacillus* with a high signal intensity. In LB cultures of *B. amyloliquefaciens* Fad 82, the signal intensity of most surfactin congeners were several orders of magnitude lower than that of the other two strains. Iturins were also produced by all three strains but the log[signal intensities] in extracts from cultures of *B. amyloliquefaciens* Fad We, and Fad 82 was about 100 times lower than that in the *B. velezensis* FUA2155 extracts. Fengycins were not detected in any of the cultures.

3.3 Antifungal activity of surfactin, fengycin, and iturin A

The minimum inhibitory concentration of commercial surfactin, fengycin and iturin A was determined with a critical dilution assay with five mycelial fungi and three yeasts as indicator strains. Iturin A inhibited 7 of the 8 indicator strains with an MIC ranging from 10 to 50 mg/L; only *S. fibuligera* was relatively resistant (Figure 2). The MIC of surfactin and fengycin against most indicator strains ranged from 300 to 500 mg/L, the highest concentration that was tested. Fengycin inhibited *M. racemosus* FUA5009 and S. *cerevisiae* FUA4002 at a concentration of about 200 mg/L.

3.4 Performance of *Bacillus* as starter cultures in simplified *daqu* model

Of the three strains of *Bacillus* applied in this study, *B. velezensis* FUA2155 was isolated from *daqu* (Wang et al., 2018), while the two strains of *B. amyloliquefaciens* were isolated from ropy bread (Röcken and Spicher, 1993). All three encode for multiple amylases, which were shown to hydrolyse starch during storage of bread (Li et al., 2020), but are beneficial technological traits in *daqu*. To determine their suitability as cultures for *daqu*, the performance of the three strains was evaluated in a simplified *daqu* model using un-inoculated wheat flour as a control. The change of the pH and the viable cell counts during the fermentation are shown in Figure 3 and Figure 4, respectively. A decrease of pH from 6.5 to lower than 5.5 was observed in the four *daqu* models, including the control. The pH increased in samples inoculated with *B. velezensis* FUA2155 during the ripening stage.

The total cell counts during the simplified *daqu* model are illustrated in Figure 4. *B. velezensis* FUA2155 and *B. amyloliquefaciens* Fad We showed high cell counts of approximately 8 log(CFU/mL) after the shaping stage and remained consistent until the end of the fermentation. Cell counts of samples inoculated with *B. amyloliquefaciens* Fad 82 dropped sharply after the ripening stage, and a similar trend was observed in the control.

An initial assessment of whether the inocula persisted relative to flour-derived microorganisms, was based on the observation of colony morphology on LB agar plates. In uninoculated control samples, colonies with a morphology that matched the strains of *Bacillus* that were used as inoculum accounted for less than 10 % of the total colonies throughout incubation. In samples inoculated with *B. amyloliquefaciens* Fad 82, about 70 % of colonies had the same morphology as the inoculum. In samples inoculated with *B. amyloliquefaciens* Fad We or *B. velezensis* FUA2155, more than 90 % of the colonies had the same morphology as the inoculum. Taken together, cell

311 counts in combination with observation of the colony morphologies and the quantification of gene
312 expression (see below) suggest that *B. amyloliquefaciens* Fad We and *B. velezensis* FUA2155 were
313 the dominant fermentation microbiota in the simplified *daqu* models.

314 In addition to bacterial growth, the growth of mycelial fungi was observed on the surface of *daqu*
315 samples (Table 3). Substantial mold growth was observed in the uninoculated control samples at
316 day 2 and mycelia covered most of the surface by day 3. Mold growth on samples inoculated with
317 *B. amyloliquefaciens* Fad 82 was comparable to the control. Mold growth and formation of conidia
318 was not detected on samples inoculated with *B. amyloliquefaciens* Fad We until day 4, while no
319 visible mold growth was observed on samples inoculated with *B. velezensis* FUA2155. On day 4,
320 samples were transferred to 55 °C which inhibited any further growth of molds irrespective of the
321 inoculum (Table 3).

322 **3.5 Gene expression of *srfAA*, *fenA* and *ituA* on the first, second, and third day of the**
323 **simplified *daqu* model**

324 To determine whether antifungal lipopeptides are expressed during the growth of *Bacillus* in the
325 simplified *daqu* model, mRNA encoding for *srfAA*, *fenA* and *ituA* was quantified in the samples
326 of simplified *daqu* model by RT-qPCR (Figure 5). Relative gene expression was calculated with
327 *gyrB* as the housekeeping gene and exponential cultures in LB broth as reference conditions. All
328 three genes were expressed by all three strains during growth in the simplified *daqu* model. Over-
329 expression of *srfAA* and *fenA* was observed at day 1 and/or day 2 of fermentation. On day 3 of
330 fermentation, *srfAA* was down-regulated in all three strains. *B. amyloliquefaciens* FAD 82 also
331 down-regulated *fenA* and *ituA* on day three of incubation (Figure 5). Taken together, the expression
332 of *srfAA*, *fenA* and *ituA* indicates that the corresponding lipopeptides may be present in *daqu*.

3.6 Antifungal activity of peptides produced by *Bacillus* in the complex *daqu* model

To provide direct evidence for production of antifungal lipopeptides in the complex *daqu* model, extracts were analysed with respect to their antifungal activity and the presence of surfactin, iturin A, and fengycin. The results of antifungal activity test of antifungal lipopeptides extracted from the complex *daqu* model are illustrated in Table 4. *A. niger* FUA5001 was used as the indicator strain to assess the antifungal activity of extracts from the complex *daqu* model. Extracts from the control samples inoculated with all 8 fermentation organisms, but not with any *Bacillus* strains, did not inhibit mold growth, indicating that bacilli were the sole or main contributors to antifungal activity. The inhibitory activity of *daqu* extracts obtained after 1 d of incubation was highly variable. Extracts from *daqu* inoculated with *B. velezensis* FUA2155 showed strong and consistent antifungal activity from the 1st to the 6th day of incubation. During this period, consistent antifungal activity was also observed for extracts obtained from *daqu* samples that were inoculated with the two strains of *B. amyloliquefaciens*. However, the inhibitory activity was weaker when compared to samples with *B. velezensis* FUA2155.

To confirm the persistence and antifungal activity of the inoculated *Bacillus* strains, gene copies representing *Bacillus*, total bacteria, and fungi in the complex *daqu* model were quantified by qPCR (Supplementary Figure S2). An increase of *Bacillus*, bacteria and fungi was found in each sample in the ripening stage (1st to 3rd day). *B. velezensis* FUA2155 showed the highest log(copies/g) of *Bacillus* and total bacteria and the lowest log(copies/g) of fungi during the whole process. The log(copies/g) of total bacteria count for *B. amyloliquefaciens* Fad We and Fad 82 from the 3rd to 10th day of fermentation ranged from 11-12 but decreased on the 13th day. Additionally, log(copies/g) of fungi remained stable after the 3rd day. Overall, the gene copies of *Bacillus*, total bacteria, and fungi were different among the complex *daqu* samples inoculated with

different *Bacillus* strains and *B. velezensis* FUA2155 showed competitive growth compared to the other two *Bacillus* strains.

3.7 Analysis of antifungal lipopeptides in extracts from the complex *daqu* model by LC-MS/MS

To further compare the production of the lipopeptides from the three strains of *Bacillus* in *daqu*, the extracts of the complex *daqu* model were also analyzed for the presence of antifungal lipopeptides. The signal intensity of the lipopeptides extracted from different time points of incubation were qualitatively detected by LC-MS/MS, and the log[signal intensity] of surfactin, iturin A, and fengycin are shown in Figure 6.

The signal intensity of surfactin, iturin A, and fengycin in the different *daqu* samples were variable. Fengycin was not detected in any of the samples. Some surfactin congeners were detected among all the samples including the control group. Because the grains used as substrate contained *Bacillus* (Figure S2), low levels of the lipopeptides can be expected (Figure 6). The signal intensity of surfactins C52-C55 in *B. amyloliquefaciens* Fad We samples from day 1 to day 10 was relatively high. Surfactin C50-C55 had the highest intensity of surfactins in *B. velezensis* FUA2155 samples from day 3 to day 10. The log[signal intensity] of iturins, on the other hand, demonstrated a substantial difference. Iturin A C47-C51 was found in all *B. velezensis* FUA2155 samples. No iturin A C47 was found in any of the samples fermented with *B. amyloliquefaciens* Fad We. The signal strength of iturins was low or below detection limit in *B. amyloliquefaciens* Fad 82 and control samples.

4 Discussion

Experimentation described in this communication expends prior knowledge by providing a comprehensive qualitative analysis of congeners of surfactin, iturin A, and fengycin from *daqu* models, as well as by documenting *in situ* antifungal activity in simple and complex *daqu* models. Previous studies discovered only one or two peptides in *jiuqu*/*baijiu* samples and did not verify their biological activity (Chen et al., 2020; Zhang et al., 2014). Moreover, current information of the contribution of the diverse microbes in *daqu* to enzymatic and microbial conversions is largely based on correlation of sequence data to metabolome data in uncontrolled, spontaneous fermentations (Deng et al., 2021; Huang et al., 2017; Xiao et al., 2017). Our study complements these past studies by targeted analysis of *daqu* models that were inoculated with one or several strains for antifungal activity and the presence of lipopeptides. Results indicate that the production of different lipopeptides by bacilli *in situ* influences the community assembly and may hence impact the flavor of *baijou*. The implications of the antifungal activity of bacilli on the quality of *baijou*, however, remain to be demonstrated in future studies.

Numerous strains of *Bacillus* spp. have been developed as biological control agents of plant and pathogens (Schirawski and Perlin, 2018). Strains of *Bacillus* occur as endophytes which produce antimicrobial peptides to protect the plants against harmful microbes (Shahzad et al., 2016). Therefore, the presence of *Bacillus* endospores in *daqu* relates to the stable occurrence of these organisms as part of commensal microbiota of plants, including wheat (Fan et al., 2011), and to the formation of spores which remain viable throughout storage and processing of grains. Correspondingly, cereal grains, flours and cereal foods generally harbor spores of *Bacillus* species (Needham et al., 2005) which makes their presence in *daqu* as well as bread predictable. The *daqu* isolate *B. velezensis* FUA2155 and the bread isolate *B. amyloliquefaciens* Fad 82 performed

399 equally well in the simple and complex *daqu* models, further indicating that the establishment
400 niche (wheat) and not the source of isolation – *daqu* versus bread – is relevant for strain selection
401 and performance.

402 Lipopeptides synthesized by *Bacillus* strains possess broad-spectrum antimicrobial activity.
403 Different lipopeptides have unique chemical structures and biological activities (Cochrane and
404 Vederas, 2016). Surfactins are powerful biosurfactants with emulsifying properties. Because of
405 the amphiphilic nature, surfactins are tightly anchored into lipid layers and can thus interfere with
406 biological membrane integrity. Surfactins have have antibacterial and antiviral abilities, but no
407 apparent antifungal effects (Ongena and Jacques, 2008).

408 Iturins exhibit strong *in vitro* antifungal activity against yeast and fungi but only limited
409 antibacterial and no antiviral activities (Aranda et al., 2005). For example, iturins from *B. pumilus*
410 HY1 inhibited *A. flavus* and *A. parasiticus* with an MIC of 50 mg/L (Cho et al., 2009). This
411 fungitoxicity of iturins has been attributed to membrane permeabilization (Gordillo and
412 Maldonado, 2012) where osmotic perturbation allows formation of ion-conducting pores as
413 opposed to the membrane disruption caused by surfactins (Aranda et al., 2005). Based on the
414 results of the MIC assays of surfactin, iturin A and fengycin against several strains of yeasts and
415 fungi in this study, iturin A showed the highest antifungal activity against 7 of the 8 indicator
416 strains with an MIC ranging from 10 to 50 mg/L (Figure 2), which is in agreement to earlier studies
417 (Carrillo et al., 2003; Cho et al., 2009).

418 The action of fengycin is less known compared with other lipopeptides but it also readily interacts
419 with lipid layers to alter the permeability of cell membranes (Deleu et al., 2005). Fengycin exhibits
420 antifungal activity, specifically against filamentous fungi (Vanittanakom et al., 1986). Recently,
421 fengycin was reported to mediate the cross-kingdom communication between bacteria and fungi

(Venkatesh et al., 2022). Fengycin facilitates bacterial invasion into fungal chlamydospores when comparing the growth of fungal strains and wild type *B. velezensis* or *B. velezensis* △*fenD* in a co-culture system.

Different lipopeptides are known to act in an antagonistic or synergistic manner. For example, surfactins showed antagonistic activity with fengycins (Tao et al., 2011). The MIC of fengycins against *Rhizopus stolonifer* increased from 0.4 to 2.0 g/L when commercial surfactins were added (Tao et al., 2011). In contrast, a synergistic effect was found to be in relation to interactions between iturin A and surfactins. With the addition of iturin A, the haemolytic activity of surfactin was significantly increased (Maget-Dana et al., 1992).

Iturins were more inhibitory than fengycins against *Gibberella zeae*, while the surfactins demonstrated no activity even at the highest concentration (1 μM) tested (Dunlap et al., 2011). In cocultures of *Trichoderma harzianum* and *B velezensis*, iturin inhibited *T. harzianum* more effectively than fengycin and surfactin (Vassilev et al., 2022). Overall, these findings, together with the MIC test and mass spectrometry analysis of *daqu* samples in this study, indicate that iturin A produced by *B. velezensis* FUA2155 is likely the main contributor for the higher antifungal effect of this strain when compared to the two other strains of *Bacillus* (Table 1) (Dunlap et al., 2011; Vassilev et al., 2022).

Bacilli produce fengycins, iturins and surfactins with a variable acyl side chain with a length of C3 to C13. Analysis of surfactins in food fermentation also indicated the present of different congeners differing in the length of the acyl side chain (Lee et al., 2012). Ribosomally synthesized lipopeptides are often specifically acylated with only one fatty acid while nonribosomally synthesized lipopeptides are usually produced as congener mixtures (Hubrich et al., 2022). Results of this study suggest that major congeners of surfactin and iturin have an acyl side chain with 10-

13 carbon atoms. To date, the formation of different congeners of lipopeptides in food fermentations has not been described. Moreover, the role of the length of the acyl side chain for the antifungal activities of different congeners are not completely understood because most available studies are confounded by the presence of multiple isoforms or multiple lipopeptides in even purified fractions (Kang et al., 2020; Kourmentza et al., 2021; Wang et al., 2017).

Production of antifungal lipopeptides has been described for multiple strains of *Bacillus* isolated from solid-state fermented products (Lee et al., 2016; Owusu-Kwarteng et al., 2020; Wu et al., 2021). The substrate for growth of bacilli strongly impacts the overall amount of lipopeptides, and the relative abundance of different lipopeptides (Hubrich et al., 2022), therefore, studies in laboratory media do not reliably predict the production of lipopeptides in food fermentations. Only few investigations, however, detected the presence of these peptides *in situ* during food fermentation (Chen et al., 2020; Zhang et al., 2014). Surfactin was produced by *Bacillus* spp. in the Moutai fermentation processes during *daqu* and stacking fermentation stages, but only a minute fraction carried over with the distillation into the final liquor (Chen et al., 2020).

In conclusion, the presence of *Bacillus* spp. in *daqu* fermentation not only affects the production of amylolytic and proteolytic enzymes in the fermentation process but also impacts the community composition assembly by production of antimicrobial lipopeptides. Because the bacterial production of both lipopeptides and of hydrolytic enzymes are dependent on the growth medium and may be dependent on a social context, studies that use complex re-constituted fermentation microbiota are necessary to further our understanding of the interaction between strains of *Bacillus* spp. and fungi and its impact on product quality.

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Figure Legends

Figure 1. Heat map of lipopeptide congeners from *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82, and *B. velezensis* in liquid LB broth samples. Samples were analyzed *via* LCMS and the log[signal intensities] are shown in a gradient. The heatmap is representative of experiments performed in triplicate biological replicates.

Figure 2. Minimum inhibitory concentration of surfactin (yellow bar), fengycin (green bar) and iturin A (blue bar) against filamentous fungi and yeasts. Results were presented as means ± standard deviation of quadruplicate independent experiments. Significant differences were determined by t-test and labeled with asterisks: *, $P < 0.05$; ** $P < 0.01$; ***, $P < 0.001$.

Figure 3. The pH during the fermentation of the simplified *daqu* model. Different line colors indicate the different strains inoculated in the *daqu* models: green, *B. amyloliquefaciens* Fad We; blue, *B. amyloliquefaciens* Fad 82; yellow, *B. velezensis* FUA2155; and gray, control (without addition of *Bacillus* strain). Different stages of the fermentation are indicated.

Figure 4. Viable cell counts during incubation of the simplified *daqu* model. Different line colors indicate different strains inoculated in the *daqu* samples: green, *B. amyloliquefaciens* Fad We; blue, *B. amyloliquefaciens* Fad 82; yellow *B. velezensis* FUA2155; and gray, no inoculation. Different stages of the incubation are indicated. Results are presented as means ± standard deviation for five biological replicates.

Figure 5. Expression of *srfAA, fenA* and *ituA* in the samples of the 1st (orange bar), 2nd (blue bar), and 3rd day (purple bar) of the simplified daqu model using *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82 and *B. velezensis* FUA2155. Relative gene expression was quantified by RT-qPCR with *gyrB* as the housekeeping gene and exponential cultures in LB broth as reference

conditions. Significant differences ($P < 0.05$) between the *daqu* model and LB broth conditions were determined by t-test and labeled with asterisks. Data represent means ± standard deviation of the means from three independent experiment.

Figure 6. (A) Heat map of lipopeptide congeners produced by *B. amyloliquefaciens* Fad We, Fad 82, and *B. velezensis* FUA2155 in the complex *daqu* model inoculated with 8 bacterial and fungal strains, and uninoculated control samples. Samples analyzed *via* LCMS and the log[signal intensities] are shown in a gradient. **(B)** Base structures of antifungal lipopeptide congeners, denoted by the varying alkyl chain lengths. The heatmap is representative of experiments performed in triplicate biological replicates.

Figures and tables

Table 1. List of strains used in this study.

Microorganism	Strains and origin	Incubation conditions	Purpose in this study	Reference
Bacillus amyloliquefaciens	Fad We; ropy bread	37 ℃, LB	Simplified and complex *daqu* fermentation	(Röcken and Spicher, 1993)
B. amyloliquefaciens	Fad 82; ropy bread	37 ℃, LB	Simplified and complex *daqu* fermentation	(Röcken and Spicher, 1993)
B. velezensis	[a] FUA2155; *daqu*	37 ℃, LB	Simplified and complex *daqu* fermentation	(Wang et al., 2018)
Kosakonia cowanii	FUA10121; *daqu*	37 ℃, LB	Complex *daqu* fermentation	(Wang et al., 2018)
Weissella cibaria	FUA3456; sourdough	30 ℃, mMRS	Complex *daqu* fermentation	
Saccharomyces cerevisiae	FUA4002; sourdough	30 ℃, MEA	Complex *daqu* fermentation and MIC test	
Saccharomycopsis fibuligera	FUA4036; *daqu* starter	30 ℃, MEA	Complex *daqu* fermentation and MIC test	
Pichia kudriavzevii	FUA4039; *daqu* starter	30 ℃, MEA	Complex *daqu* fermentation and MIC test	
Aspergillus niger	FUA5001	25 ℃, MEA	Complex *daqu* fermentation and MIC test	(Black et al., 2013; Liang et al., 2020)
Mucor racemosus	FUA5009	25 ℃, MEA	Complex *daqu* fermentation and MIC test	
Penicillium roqueforti	FUA5012	25 ℃, MEA	Complex *daqu* fermentation and MIC test	
A. clavatus	FUA5004	25 ℃, MEA	MIC test	
A. clavatus	FUA5005	25 ℃, MEA	MIC test	

[a] FUA number, Food microbiology culture collection at the University of Alberta.

Table 2. List of primers used in RT-qPCR.

Target	**Primer**	**Sequence (5'-3')**
gyrB	*gyrB* Forward	ATCGTCGACAACAGTATTG
	gyrB Reverse	CTTTATATCCGCTTCCGTC
srfAA	*srfAA* Forward	GACAAGCGGCGTCATCAATC
	srfAA Reverse	CTGCCACGCATAATTCACCG
fenA	*fenA* Forward	TGCGGTTAACGGCAAACGG
	fenA Reverse	TCAAGAAGCCATTCAGTTCGCG
ituA	*ituA* Forward	CCGGCACGATTGATATCGC
	ituA Reverse	CCGGCCTGCTTGATAAAGC

Table 3. Degree of the mold growth during the first 4 days of the simplified *daqu* model.

Inoculated strain	**Incubation time**				
	Day 0	Day 1	Day 2	Day 3	Day 4
Control	-	-	+++	++++	+++++
B. amyloliquefaciens Fad We	-	-	+	+	++
B. amyloliquefaciens Fad 82	-	-	++++	++	+++
B. velezensis FUA2155	-	-	-	-	-

–, no mycelial growth visible; +, small spots of mycelial growth; ++, spots of mycelial growth and conidia; +++, 25-50 % of the surface covered by mycelium; and ++++, more than 50 % of the surface covered by mycelium. The reference pictures for defining the fungal growth are indicated in the Figure S1.

Table 4. Antifungal activity of peptides extracted from the complex *daqu* model with different strains of *B. amyloliquefaciens* or *B. velezensis*.

	Incubation time					
Inoculated strain	Day 1	Day 3	Day 6	Day 10	Day 13	Day 16
Control	0	0	0	0	0	0
B. amyloliquefaciens Fad We	3 ± 4	1 ± 1	1 ± 0.5	1 ± 0.5	0 ± 0	0.3 ± 0.5
B. amyloliquefaciens Fad 82	0.3 ± 0.5	2 ± 1.7	1 ± 0.8	1 ± 0	1 ± 0	2 ± 2
B. velezensis FUA2155	3 ± 4	3 ± 1	2 ± 1	1 ± 0	1 ± 0.5	1 ± 2

0: no inhibition; 0-0.9: slight inhibition; 1-1.9: moderate inhibition; 2-2.9: strong inhibition; ≥ 3: significant inhibition. Data represent means ± standard deviation of the means from three independent experiment. Experiments were down in triplicate.

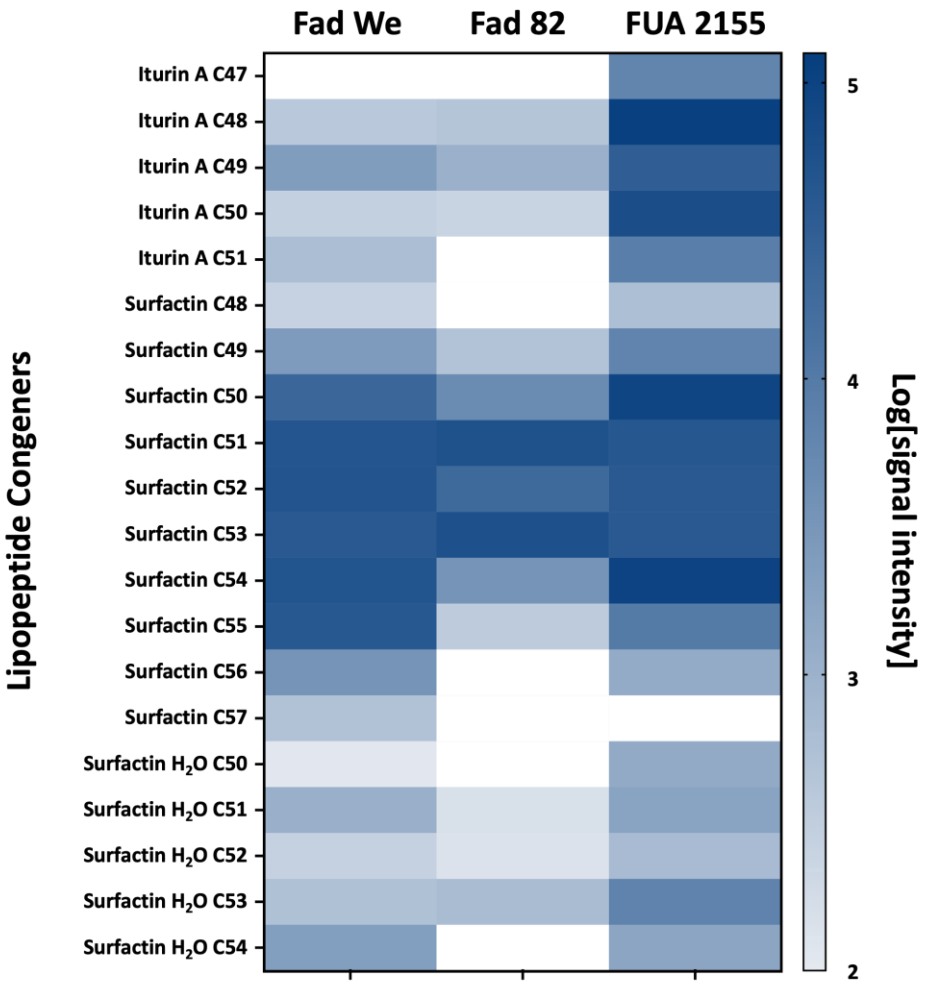
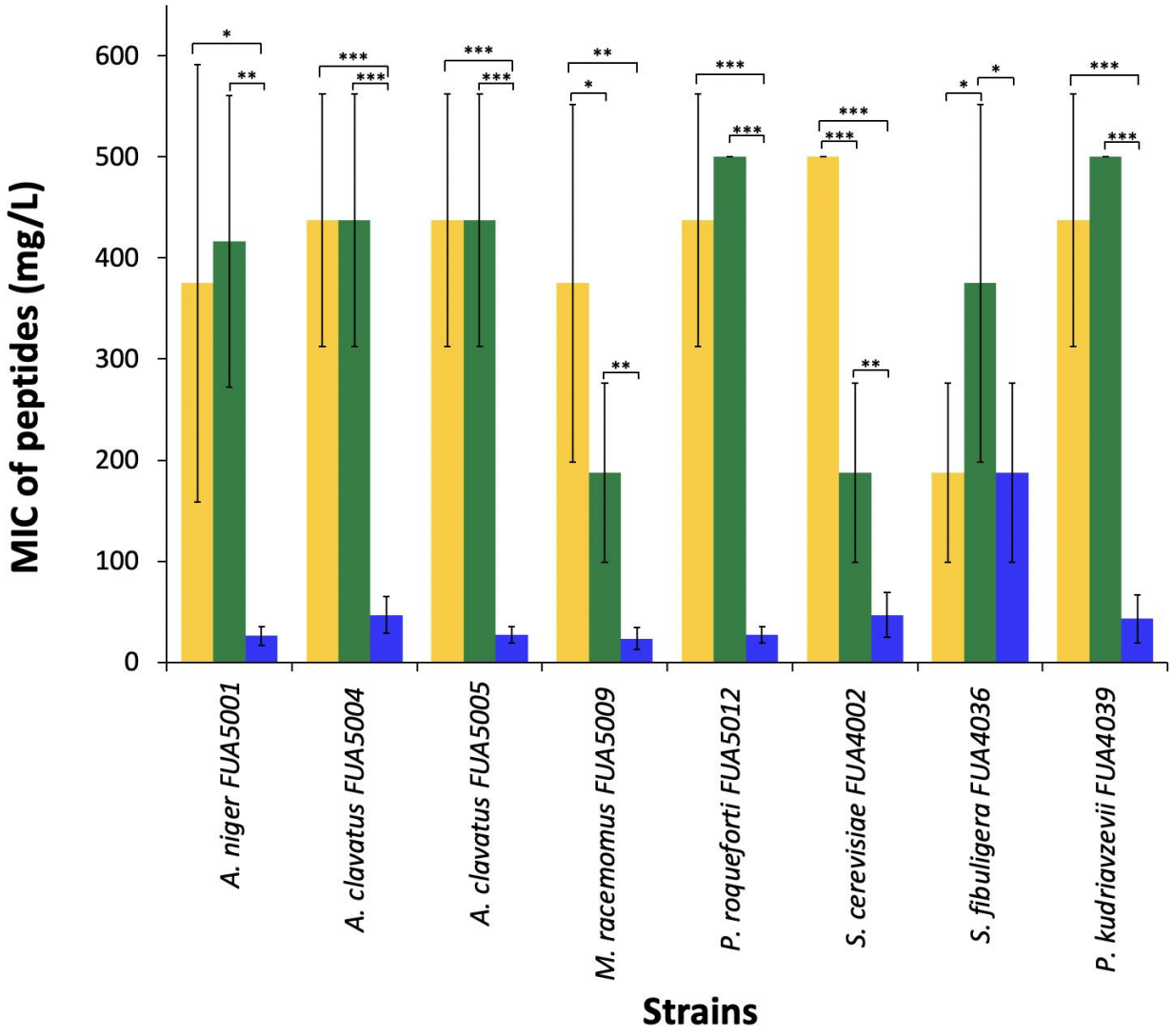

Fad We
Fad 82
FUA 2155
Iturin A C47
Iturin A C48
Iturin A C49
Iturin A C50
Iturin A C51
Surfactin C48
Surfactin C49
Surfactin C50
Surfactin C51
Surfactin C52
Surfactin C53
Surfactin C54
Surfactin C55
Surfactin C56
Surfactin C57
Surfactin H2O C50
Surfactin H2O C51
Surfactin H2O C52
Surfactin H2O C53
Surfactin H2O C54
Lipopeptide Congeners
Log[signal intensity]
5
4
3
2

Figure 1.


MIC of peptides (mg/L)
600
500
400
300
200
100
0
A. niger FUA5001
A. clavatus FUA5004
A. clavatus FUA5005
M. racemomus FUA5009
P. roquefortii FUA5012
S. cerevisiae FUA4002
S. fibuligera FUA4036
P. kudriavzevii FUA4039
Strains

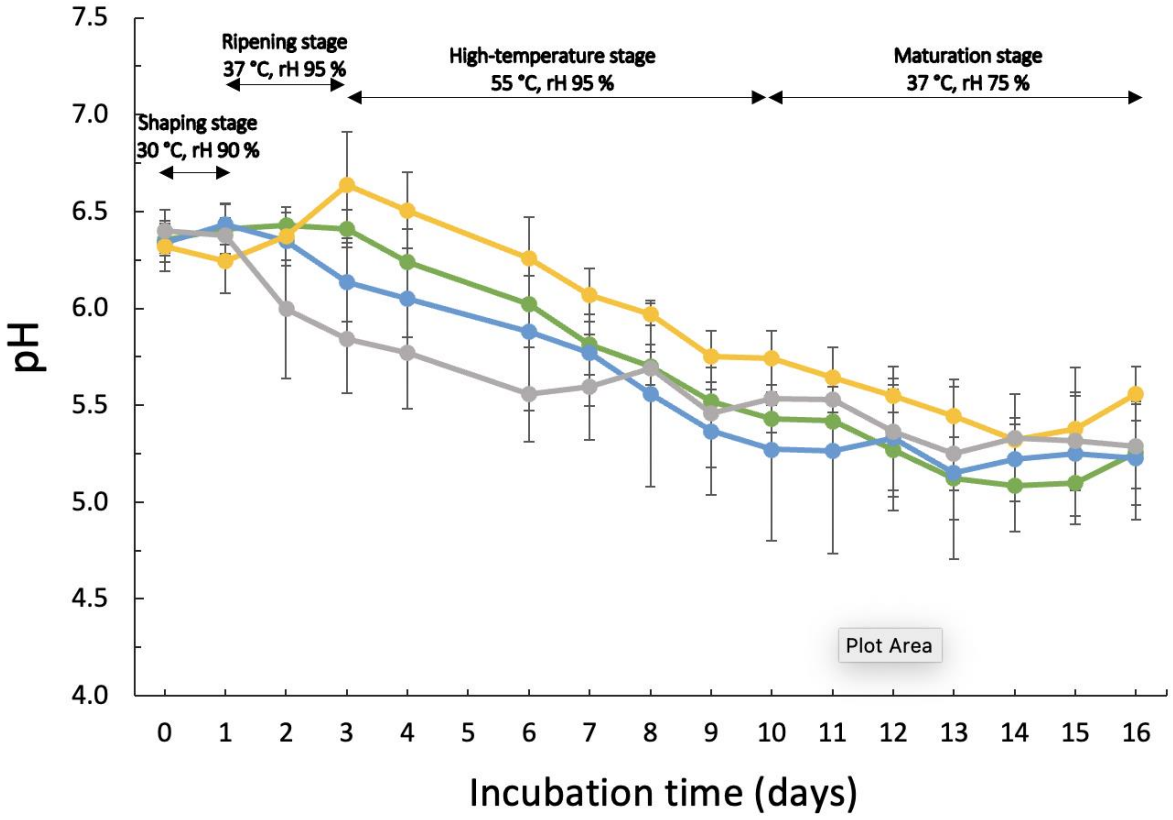
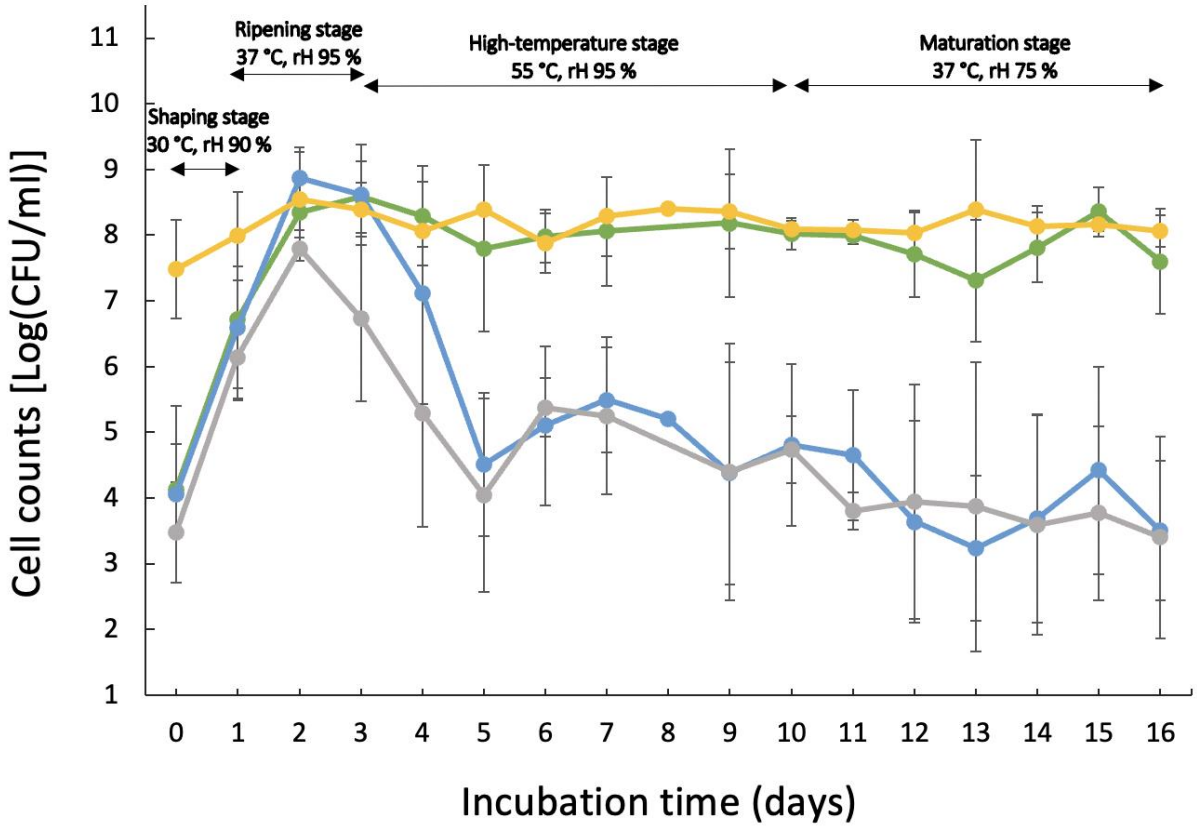

Ripening stage
37 °C, rH 95 %
High-temperature stage
55 °C, rH 95 %
Maturation stage
37 °C, rH 75 %
Shaping stage
30 °C, rH 90 %
pH
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
Plot Area
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Incubation time (days)

Figure 3.


Ripening stage
37 °C, rH 95 %
High-temperature stage
55 °C, rH 95 %
Maturation stage
37 °C, rH 75 %
Shaping stage
30 °C, rH 90 %
Cell counts [Log(CFU/ml)]
Incubation time (days)
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

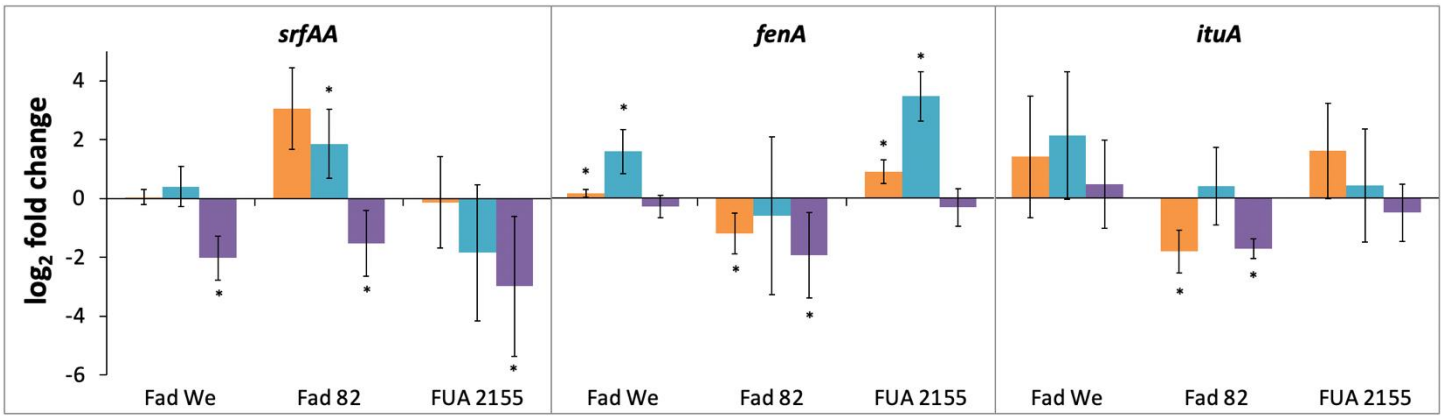

srfAA
fenA
ituA
log2 fold change
4
2
0
-2
-4
-6
Fad We
Fad 82
FUA 2155
Fad We
Fad 82
FUA 2155
Fad We
Fad 82
FUA 2155

Figure 5.

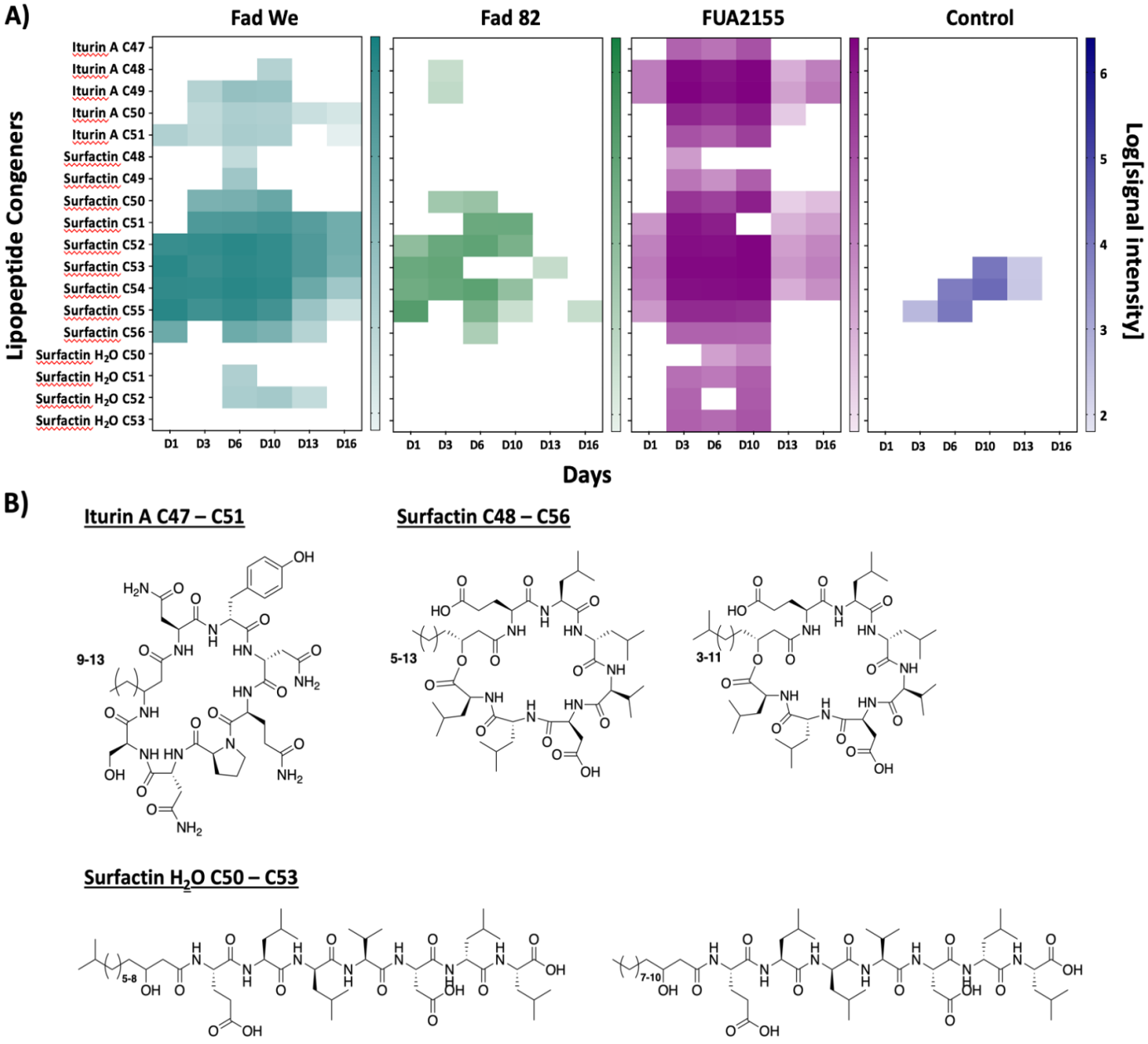

A)
Fad We
Fad 82
FUA2155
Control
Lipopeptide Congeners
Iturin A C47
Iturin A C48
Iturin A C49
Iturin A C50
Iturin A C51
Surfactin C48
Surfactin C49
Surfactin C50
Surfactin C51
Surfactin C52
Surfactin C53
Surfactin C54
Surfactin C55
Surfactin C56
Surfactin H2O C50
Surfactin H2O C51
Surfactin H2O C52
Surfactin H2O C53
D1 D3 D6 D10 D13 D16
Days
Log[signal intensity]
2 3 4 5 6
B)
Iturin A C47 – C51
9-13
Surfactin C48 – C56
5-13
3-11
Surfactin H2O C50 – C53
5-8
7-10

Figure 6.

Online supplementary material to

Composition and activity of antifungal lipopeptides produced by *Bacillus* spp. in *daqu* fermentation

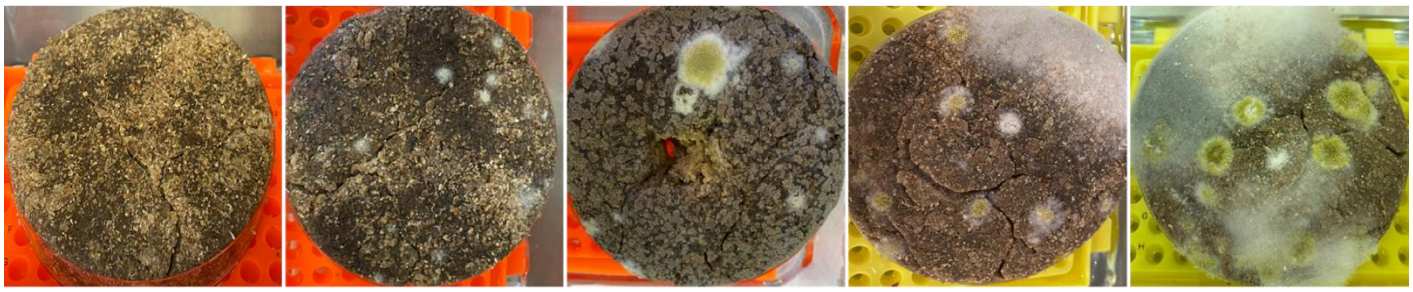
Zhen Li, Kleinberg X. Fernandez, John C. Vederas, Michael G. Gänzle

Supplementary Figure S1. Reference pictures for the designation of fungal growth during the simplified *daqu* fermentation model.

Supplementary Figure S2. Microbiota analysis by qPCR during the fermentation of the complex *daqu* model samples. **(A** *Bacillus*. **(B)** Total bacteria. **(C)** Fungi. Different color of the line indicated different strains inoculated in the *daqu* fermentation: green, *B. amyloliquefaciens* Fad We; blue, *B. amyloliquefaciens* Fad 82; yellow *B. velezensis* FUA2155; and gray, no inoculation. The data was based on three replicates of qPCR for one DNA isolation.

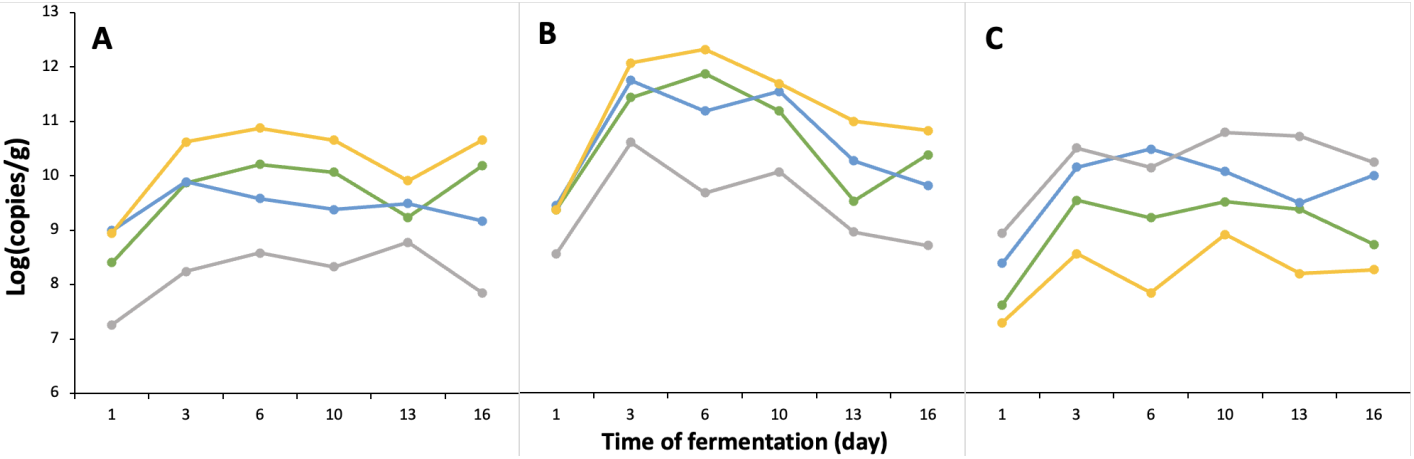
Supplementary Table S1. Prediction of cluster of different antifungal peptides in the genome of three strains of *Bacillus* predicted by antiSMASH.

Supplementary Table S2. Identification of gene clusters encoding different lipopeptides by BLAST.

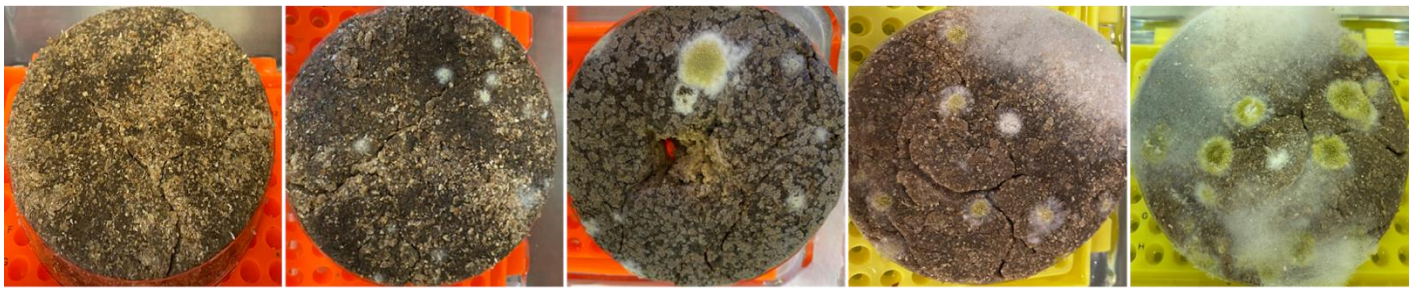


- + ++ +++ ++++

Supplementary Figure S1 Reference pictures for the designation of fungal growth during the simplified *daqu* fermentation model: –, no mycelial mold growth visible; +, small spots of mycelial growth; ++, spots of mycelial growth and conidia; +++, 25-50 % of the surface covered by mycelium; and ++++, more than 50 % pf the surface covered by mycelium.



Supplementary Figure S2. Microbiota analysis by qPCR during the fermentation of the complex *daqu* model samples. **(A)** *Bacillus*. **(B)** Total bacteria. **(C)** Fungi. Different color of the line indicated different strains inoculated in the *daqu* fermentation: green, *B. amyloliquefaciens* Fad We; blue, *B. amyloliquefaciens* Fad 82; yellow *B. velezensis* FUA2155; and gray, no inoculation. The data was based on three replicates of qPCR for one DNA isolation.



- + ++ +++ ++++

Supplementary Figure S2 Reference pictures for the designation of fungal growth during the simplified *daqu* fermentation model: –, no mycelial mold growth visible; +, small spots of mycelial growth; ++, spots of mycelial growth and conidia; +++, 25-50 % of the surface covered by mycelium; and ++++, more than 50 % pf the surface covered by mycelium.

Supplementary Table S1. Prediction of cluster of different antifungal peptides in the genome of three strains of *Bacillus* predicted by antiSMASH.

Secondary Metabolite	Type	Cluster	Similarity		
			Fad We	Fad 82	FUA2155
surfactin	[a]NRP:Lipopeptide	[b]NRPS	82 %	82 %	47 %
surfactin	NRP:Lipopeptide	NRPS	-	-	< 30 %
surfactin	NRP:Lipopeptide	NRPS	-	-	39 %
fengycin	NRP	NRPS, transAT-[c]PKS, betalactone	93 %	93 %	93 %
iturin	NRP, Polyketide	NRPS	77 %	77 %	88 %
bacillaene	transAT-PKS, NRPS, T3PKS	Polyketide + NRP	100 %	100 %	100 %
butirosin A/B	PKS-like	Saccharide	< 30 %	< 30 %	< 30 %
bacilysin	other	Other	100 %	100 %	100 %
bacillibactin	RiPP-like, NRPS	NRP	100 %	100 %	100 %
difficidin	transAT-PKS	Polyketide + NRP	-	-	100 %
Macrolactin H	transAT-PKS	Polyketide	-	-	100 %

[a]NRP: non ribosomal peptides

[b]NRPS: non ribosomal peptide synthetase

[c]PKS: polyketide synthetases

Supplementary Table S2. Identification of gene clusters encoding different lipopeptides by BLAST.

Lipopeptides	Accession number	Fad We		Fad 82		FUA2155	
		Query Cover	Per. Ident	Query Cover	Per. Ident	Query Cover	Per. Ident
surfactin	AJ575642.1	91 %	92.89 %	91 %	92.89 %	40 %	98.32 %
fengycin	CP000560.1	56 %	92.83 %	56 %	92.83 %	54 %	97.96 %
iturin	AB050629.1	96 %	90.14 %	96 %	90.14 %	86 %	97.90 %
bacillaene	AJ634060.2	100 %	92.47 %	100 %	92.47 %	100 %	97.98 %
butirosin A/B	AB097196.1	No significant similarity found					
bacilysin	CP000560.1	56 %	92.83 %	56 %	92.83 %	54 %	97.96 %
bacillibactin	AL009126.3	18 %	76.56 %	18 %	76.51 %	29 %	76.36 %
difficidin	AJ634062.2	No significant similarity found		No significant similarity found		100 %	97.88 %
macrolactin H	AJ634061.2	No significant similarity found		No significant similarity found		100 %	98.18 %