Staphylococcus aureus artificially inoculated in mealworm larvae rearing chain for human consumption: Long-term investigation into survival and toxin production

Survival and toxins formation of *Staphylococcus aureus* artificially inoculated in mealworm larvae rearing chain for human consumption

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Abstract

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1. Introduction

Edible insects represent a novel source of sustainable food and feed (Penazzi et al., 2021). Indeed, since 2015, a list of edible insects showing the potential to be used as food and feed in the European Union (EU) has been issued by the European Food Safety Authority (EFSA), including: the common housefly, the black soldier fly, the mealworm, the giant mealworm, the lesser mealworm, the greater wax moth, the lesser wax moth, the silkworm, the house cricket, the banded cricket, the African migratory locust, and the American grasshopper (EFSA Scientific Committee, 2015). Moreover, the inclusion of edible insects in the so-called novel food category, established by Regulation (EU) No. 2015/2283, gave a further boost to the development of newly funded companies producing food ingredients based on the use of such mini livestock.

In the EU, three insects intended for human consumption are now authorized, namely: locusts, crickets, and mealworms. Of note, the authorization for the commercialization conferred by the European Commission has to be obtained by the producer that must provide an exhaustive dossier reporting the safety assessment of the proposed insect-based food (Regulation (EU) No. 2015/2283).

In such context, the research sector can help the food and feed industry in obtaining novel insight into the risks that could be associated with the edible insect production chain, whether insects are used as feed, or they are intended for human consumption.

It is known that edible insects can be vectors of microorganisms that can be contained in the gut or spread on the external cuticle (Garofalo et al., 2019). Microbial contamination of insects is the result of vertical transmission from the mother to the offspring via egg smearing (Cesaro et al., 2022). Moreover, insects can be contaminated by microorganisms naturally occurring in the feed or in the rearing environment (Osimani et al., 2018).

The knowledge on the microbial species that could occur the insect rearing chain could help the food industry in implementing preventive actions to limit the risks for consumers. To achieve this goal, a different approach can be applied; on the one hand, studies on the microbiota naturally occurring in the insects rearing chain can provide a general overview of potential biological risks, on the other hand, the deliberate inoculation of foodborne pathogens can provide information on the risks associated with the presence of specific microorganisms. This latter approach can be pursued through the implementation of challenge tests, as already successfully tested on edible insects by other authors (Cesaro et al., 2022; Erickson, Islam, Sheppard, Liao, & Doyle, 2004; Liu, Tomberlin, Brady, Sanford, & Yu, 2008; Mancini et al., 2019; Belleggia et al., 2020; Wynants et al., 2019; Gorrens et al., 2021; De Smet et al., 2021).

As reported by Wynants et al. (2019), many factors can affect the survival and multiplication of foodborne pathogens in the insect rearing chain, including the response of the immune system of the host, the interactions among microbial populations occurring in the gut of larvae (e.g., competitive exclusion by the endogenous larval microbiota), and insect species (e.g., antibacterial activity of the larvae) (Wynants et al., 2019).

As reported by Vandeweyer, De Smet, Van Looveren, & Van Campenhout (2021), the top three of the bacterial pathogens associated with insects for food are represented by pathogenic clostridia, pathogenic species of *Bacillus*, and *Staphylococcus aureus*.

Among these microorganisms, *S. aureus* is a spherical non-spore-forming Gram-positive bacterium with an optimum growth temperature that ranges from 35 to 41 °C. *S. aureus* can be the causative agent of foodborne intoxication due to the production of enterotoxins that are synthesized by the microorganism at an optimum temperature range comprised between 35 to 41 °C. Once produced in the food, enterotoxins reach the human digestive tract and are resorbed into the blood causing nausea and/or vomiting (Bencardino, Amagliani, & Brandi, 2021). Of note, heat treatment for 60 min at 60 °C inactivates *S. aureus* cells, whereas enterotoxins produced by *S. aureus* are extremely heat resistant and, once preformed in the food, cannot be inactivated by common thermal treatments as boiling, steaming, oven cooking, or pan frying (Bencardino et al., 2021). Staphylococcal food poisoning can result from cross-contamination from food to other foods and/or non-food elements, including surfaces and workers (Bencardino et al., 2021).

To date, many published studies are available in the scientific literature on the occurrence of pathogens in edible insects; among these, only two papers deal with the dynamics of *S. aureus* along a short-time edible insects rearing period (Gorrens et al., 2021; McGonigle, Purves, & Rolff, 2016). However, as elucidated by McGonigle et al. (2016), the practice of studying short-term persistence of microorganisms in edible insects could be misleading; hence long-term studies were recognized by these authors as a more effective strategy to evaluate the fate of microorganisms in whole living period of reared insects.

In the present study, the dynamics of *S. aureus* were investigated throughout the whole living period of mealworm (*Tenebrio molitor*) larvae, from eggs to pupae. To this end, a rearing substrate consisting of organic

wheat middlings was spiked with *S. aureus* at three initial contamination levels (low, medium, and high). Microbial viable counting coupled with metataxonomic analyses were performed to evaluate: i) the persistence and growth of *S. aureus* in the rearing substrate; ii) the colonization and growth of *S. aureus* in the insect larvae; iii) the occurrence and load of *S. aureus* in the frass (excrement from larvae mixed with substrate residues); and the presence of *S. aureus* enterotoxins in the rearing substrate (or frass) and larvae.

2. Materials and methods

2.1. Experimental design

Organic wheat middlings were placed in 15 sterile boxes. In 12 of these boxes, *S. aureus* was inoculated at different contamination levels, namely 1 (low-level), 5 (medium-level) and 7 (high-level) log colony forming unit (cfu) per gram. The remaining three (C_1, C_2, C_3) were not inoculated since the aim of such boxes was to monitor the *T. molitor* development from eggs to pupae. For each contamination level, four replicates were set up (F_1, F_2, F_3, F_C). In more detail, F_1, F_2 and F_3 boxes contained organic wheat middling and *T. molitor* eggs, whereas F_C did not contain eggs since it is used only to evaluate the *S. aurues* trend in the rearing substrate during the entire experiment. Prior to the inoculum, wheat middlings and eggs were analysed through microbiological viable counts for verifying the absence of the target microorganism. Samples of eggs, larvae, wheat middlings and frass were collected at regular intervals (every 14 days), starting from the day of microorganism inoculum (t_0). Afterwards, each sample was analysed through culture-dependent techniques for the *S. aureus* microbiological viable counts. The trial was interrupted after 70 days since larvae turned into pupae. Finally, RNA of eggs, larvae, rearing substrates and frass samples were extracted and sent for sequencing.
The experimental design is represented in Figure 1.

2.2. Inocula preparation

Two different *S. aureus* strains were used. Both were separately cultured in 10 mL of Brain Heart Infusion (BHI) broth (Sigma, Milan, Italy) at 37°C for 24 hours, then they were sub-cultured in 30 mL of BHI and incubated at 37°C for other 24 hours. The biomasses of the last sub-cultures were centrifugated using a Rotofix 32A centrifuge (Hettich, Tuttlingen, Germany) at 4000 rpm for 10 minutes. Once removed the resulting supernatants, cell pellets were resuspended in physiological solution (0,85% NaCl) and optical density (OD) was measured with a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), considering that an OD_{600} of 1.0 corresponds to approximately 9 log cfu mL^{-1} (Chang et al., 2013). Then, microbiological viable counts were performed on BHI agar to evaluate cell viability. Finally, three bacterial suspensions per strain were prepared by diluting the initial suspension with physiological solution to reach the three contamination levels required: 1, 5 and 7 log cfu mL^{-1}.

2.3 Staphylococcus aureus inoculation

For the experimental test, 680 g of organic wheat middling, used as rearing substrate, were placed into all the boxes. The inoculum was set to not exceed 1% (v w^{-1}) of the assay unit to limit any modification in the rearing substrate properties (Belleggia et al., 2020). Suspensions with the two *S. aureus* strains (1:1 ratio, 3.4 mL of suspension for each strain) were inoculated in the rearing substrates: four boxes with about 1 log cfu g^{-1}, four boxes with 5 log cfu g^{-1} and the last four boxes with a microbial load of 7 log cfu g^{-1}. For each inoculum, 170 droplets (20 µL each) were distributed on wheat middlings using a semiautomatic pipette. Then, the inoculated rearing substrate was mixed using a sterile spoon. To verify the amount of each inoculum, five grams of the wheat middlings were immediately collected from all the replicates (t_0). In addition, to allow the same moisture of the inoculated trays to be reached, 6.8 mL of physiological water solution were added to the three control trays containing *T. molitor* eggs but without the target microorganism.

2.4 Mealworm larvae rearing and sampling

Approximately four kilograms of mealworms were purchased from INEF - Insect Novel Ecologic Food factory (Padova, Italy) with the aim to set up the mother colony, which was fed organic wheat middling in a climate-controlled chamber maintained at 28 ± 0.5 °C and a relative humidity of 60 ± 0.5%. Once larvae were turned into pupae, they were collected, sexed, and divided according to their sex until the reaching of the adult stage. Finally, twenty adults (sex ratio 1:1) were placed in plastic trays with organic wheat middling and filter paper

discs to favour the adhesion of the eggs. Then, 1000 eggs (corresponding to around one gram) were placed into all the boxes inoculated (F_1, F_2, and F_3), with the exception for F_C boxes, and the control trials (C_1, C_2, C_3). All the experimental batches were put inside the climate-controlled chamber as described above. As a source of water for larvae, every two days ten grams of washed and pelled carrots slices were put in all the boxes (Osimani et al., 2018).
Aliquots of wheat middlings, larvae and frass were collected from each batch every fourteen days. Prior to analysis, larvae were frozen at -20° C for 40 minutes, washed with a 70% ethanol solution in sterile deionized water (Wynants et al., 2019) and then homogenized in a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy). Larvae were collected starting from t_2 (about twenty-eight days later after start of the trial) because of their minute dimensions. Regarding frass samples, they were collected in sterile bags for microbiological viable counts (5 g) and for water activity measurements (5 g).
Larvae were constantly monitored to assess their viability and absence of visible diseases (data not shown).

2.5. Water activity measurement

During sampling, the water activity (a_w) of wheat middlings and frass was measured according to the ISO 21807:2004 standard method using an AwTherm apparatus (Rotronic, Bassersdorf, Switzerland).

2.6. Bacterial counts

Five-gram aliquots of wheat middlings and frass were suspended in 45 mL of sterile peptone water (1 g L^{-1} bacteriological peptone), whereas 25 larvae, which were collected starting from t_2, were weighted at each sampling, crushed under aseptic conditions (Vandeweyer et al. 2019) and added to a suitable amount of sterile peptone water to perform the ten-fold serial dilutions for microbiological viable counts. A Stomacher apparatus (International PBI, Milan, Italy) was used for 1 minutes at 260 rpm for the sample homogenization. The final suspensions were ten-fold diluted in sterile peptone water, plated on Baird Parker agar base (BP) medium (VWR, Leuven, Belgium) supplemented with Egg Yolk Tellurite Emulsion (VWR, Leuven, Belgium) and incubated at 37° C for 24 hours to enumerate *S. aureus*. The results were expressed as the log cfu g^{-1} and reported as mean ± standard deviation (Table 1).

2.7. RNA extraction and cDNA synthesis

From each sample homogenate, 1.5 mL aliquots (dilution 10^{-1}) were centrifuged for 10 minutes at 16,000 rpm; the resulting supernatants were discarded, and the cell pellets were covered with RNAlater Stabilization Solution (Ambion, Foster City, CA, USA) and stored at -80 °C until use. Prior to RNA extraction, pellet samples of the same contamination level and sampling time were pooled (Ezeokoli, Gupta, Mienie, Popoola, & Bezuidenhout, 2016).
Microbial RNA was extracted through an E.Z.N.A. Bacterial RNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. The extracted RNAs were checked for the presence of residual DNA by PCR amplification using the universal prokaryotic primer pair 27f and 1495r (Weisburg, Barns, Pelletier, & Lane, 1991). Moreover, RNAs were also checked for quantity, purity, and integrity as described by Garofalo et al. (2017). cDNA synthesis was performed using the SensiFAST cDNA Synthesis Kit (Bioline, London, UK) in accordance with the manufacturer's instructions.

2.8. Metataxonomic analysis

To investigate the microbiota composition and evaluate the presence of the inoculated *S. aureus* among larvae, wheat middlings, frass, and egg samples, a metataxonomic approach was applied. The protocol described by Klindworth et al. (2013) was used to amplify the V3-V4 region of the 16S rRNA gene of the cDNA samples. The obtained amplicons were purified, tagged, and pooled following Illumina procedures. Hence, 250 bp paired-end reads were constructed by using the MiSeq platform (Illumina), and the corresponding raw files (FASTQ format) were imported into QIIME2 software (Bolyen et al., 2019). In QIIME2 software, barcode sequences and primers were eliminated, and the resulting sequences were denoised through DADA2 package to acquire the amplicon sequence variants (ASVs) (Callahan et al., 2016). A total of 274,834 reads were used for the downstream analysis, with an average of 4,658 sequences per sample and a sample coverage > 99%. The taxonomy assignment was executed in QIIME2 by means of SILVA database v13.8. Filtering of ASVs

with a relative frequency > 1.0% in at least two samples was performed. To verify the taxonomic assignment of each ASV, a comparison with 16S rRNA sequences of GenBank database (http://www.ncbi.nlm.nih.gov/) was performed by Basic Local Alignment Search Tool (BLAST). Metataxonomic analysis sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (PRJNA839909).

2.9. Detection of staphylococcal enterotoxins

The detection of staphylococcal enterotoxins was performed according to UNI EN ISO 19020:2017, following a protocol divided in two steps: Extraction/concentration and Immuno-enzymatic detection performed using the VIDAS® equipment with Staph enterotoxin II (SET2) kit (bioMérieux, Marcy-l'Étoile, France).
Briefly, 10 gr of each sample were mixed with 40 mL of warm (38°C ± 2 °C) distilled water in total filter-bags and homogenized by stomacher. The sample was left 40 min at room temperature and shaken every 10 min to allow any toxin to diffuse.
Afterwards, the mixture was collected, acidified to pH of 3.5-4.0 adding hydrochloric acid and centrifuged at 3175 g for 15 min at 4°C. The supernatant was then recovered, neutralized using sodium hydroxide to a pH value between 7.4-7.6, then centrifuged again. Next, the neutralized aqueous phase was collected for the subsequent dialysis-concentration step.
To this purpose, a dialysis membrane with 7000 Daltons MWCO (SnakeSkin Dialysis Tubing, ThermoScientific, Rockford, USA), was filled with the neutralized aqueous phase, previously filtered through cotton wool, and placed in a 30% (w/v) PEG solution over night at 4°C. The concentrated extract was recovered in 2 mL distilled water rinsing the internal wall of the dialysis tube several times in order to recover the maximum amount of material. The SET2 assay was performed immediately after according to manufacturer's instruction, testing 500 µL of extract. This kit detects, without differentiation, the Staphylococcal Enterotoxins (SE) A, B, Cs, D and E. The test value (TV) of the sample is automatically calculated by Vidas® system (bioMérieux) and represents the ratio between Relative Fluorescence Value (RFV) of the sample and the RFV of the standard solution. Samples that give TV equal or greater than 0.13 are considered as positive.

2.10. Statistical analysis

After first checking for conformance to a normal distribution, Tukey–Kramer's honest significant difference (HSD) test (level of significance 0.05) was used to evaluate differences in viable counts and physico-chemical parameters within samples by one-way analysis of variance (ANOVA). Tests were carried out using JMP Version 11.0.0 software (SAS Institute Inc., Cary, NC).
ASV tables, filtered at 0.5% abundance in at least two samples, were used for co-occurrence/co-exclusion analysis carried out by the psych package of R (www.r-project.org). Only significant correlations (FDR < 0.05) were visualized by using the corrplot package of R.

3. Results

3.1. S. aureus counts

Viable counts carried out in all the experimental trays are reported in Table 1.
The counts of *S. aureus* in the eggs of the insect, in the wheat middlings before inoculation, and in larvae reared on the uninoculated wheat middlings (from t1 to t5) showed values < 1 log cfu g^{-1}.
Viable counts of *S. aureus* carried out in the rearing substrates sampled at t0 after inoculation indicated that the required contamination levels (1, 5, and 7 log cfu g^{-1}) were achieved in all the trays.
The inoculated wheat middlings without larvae, used to test the persistence of the pathogen in the rearing substrate, showed a progressive decrease of viable cells of *S. aureus*, depending on the initial inoculation level. In frass samples, after t1 (14 days), a statistically significant reduction in *S. aureus* living cells was observed in all frass samples, except for those collected in the low-level contamination samples. In more detail, from t2 to t3, *S. aureus* levels were lower than 1 log cfu g^{-1} in wheat middlings initially spiked with 1 log cfu g^{-1}, whereas at t4 and t5 the counts showed values reaching approximately 1 log cfu g^{-1}. In frass samples collected in the medium-level contamination, the counts of *S. aureus* attested at 0.53 log cfu g^{-1} at t5. Finally, in the high-level contamination, the counts of *S. aureus* in frass samples collected at t5 attested at 2.04 log cfu g^{-1}.

Regarding samples of larvae, the presence of the target microorganism was never detected at any sampling time starting from t2, irrespective of the contamination level.
A_w values detected in all the analyzed samples (wheat middlings or frass) are reported in Table 1. In more detail, the a_w values recorded at t0 in the uninoculated wheat middlings used as control attested at 0.54, with final values comprised between 0.52 and 0.55 in all wheat-based substrates (wheat middlings or frass).

3.2. Metataxonomic analysis

3.2.1. Control experiments

Supplementary Table 1 shows the relative frequency of each microbial group detected by metataxonomic analysis in *T. molitor* egg samples. *Staphylococcus epidermidis* represented the major ASV with 54.10% of relative frequency. *Acidiphilium*, *Enterococcus*, *Sphingomonas*, *Paracoccus*, and *Bacillus* followed with 14.54, 8.53, 4.20, 2.20, and 1.03% of relative frequency, respectively.
The metataxonomic analysis results of larvae reared on uninoculated wheat middlings are listed in Table 2 (Panel a). *Staphylococcus* was absent at t_2, whereas it registered an increase during time up to 38.97% of the relative frequency. *Weissella*, *Streptococcus*, and *Rhizobium* also showed the same trend, and reached 14.64, 12.55, and 10.72% at t_5, respectively. The taxa *Acinetobacter* and *Exiguobacterium* were dominant at t_2 with 58.64 and 36.34% of the relative frequency, respectively, and strongly decreased over time.
Table 2 showed the microbial group frequency of uninoculated wheat middlings and frass of larvae rearing (Panel b). *Staphylococcus* was absent at the beginning of the experiment, whereas an increase in the other sampling times was highlighted, with 7.51% of the relative frequency at t_5. *Pantoea* and *Pseudomonas*, the most representative groups at t_0, decreased up to 2.83 and 9.03% at t_5, respectively. The microbial taxa detected at t_5 resulted heterogeneous, with *Paracoccus* and *Sphingomonas* as the predominant ones (10.75 and 10.68%, respectively). Besides, an increase of the detected ASVs was observed during time.
Co-occurrence/co-exclusions analysis showed that *Acidiphilium* co-occurred with *Sphingomonas* and *Bacillus* while *Sphingomonas* co-occurred with *Pantoea* and *Pseudomonas.* No associations were observed between *Staphylococcus epidermidis* or *S. aureus* and the microbiota (data not shown).

3.2.2. Low-level contamination experiments

The bacterial taxonomic groups of larvae reared on wheat middlings inoculated with 1 log cfu *S. aureus* g^{-1} are shown in Table 3 (Panel a). *Staphylococcus* was not detected or slightly detected until t_4 and registered an increase at t_5 up to 5.86% of the relative frequency. The microbial taxa *Enterobacter*, *Streptococcus*, and *Peptoniphilus* increased over time, reaching 21.26, 10.54, and 7.09% at the end of the experiment. The genus *Acinetobacter* was predominant at t_2 with 96.72% of the relative frequency, whereas it showed 8.54% at t_5. *Moraxella* and *Bacillus* displayed an irregular pattern during time, as they were detected among the most representative groups at t_3 and t_5.
Panel b of Table 3 reports the microbial group frequencies of wheat middlings (t0) and frass of larvae rearing inoculated with 1 log cfu *S. aureus* g^{-1}. *Staphylococcus* was present at the start of the experiment with 11.76% of the relative frequency, reached 28.89% at t_3, and was not detected at t_5. A stable trend was highlighted for the genus *Streptococcus* with values around 25%. *Veillonella*, *Moraxella*, and *Gemella* registered an increase over time, with 15.39, 15.34, and 13.53% of the relative frequency at the end of the experiment. Conversely, *Pseudomonas* decreased up to 4.56% obtained at t_5. The genus *Acinetobacter* was the major taxonomic group detected at t_2 with 29.47% of the relative frequency, whereas resulted absent at t_5.
Regarding the control wheat middlings inoculated with 1 log cfu *S. aureus* g^{-1}, data are shown in Panel c of Table 3. *Staphylococcus* was present at the start of the experiment with 11.76% of the relative frequency and increased up to 35.42% at the end. As for *Streptococcus*, such genus registered variable values during time, with about 5% of the relative frequency at t_0 and t_5. *Moraxella* relative frequency increased up to 49.88% at t_4, whereas showed 16.68% at the end of the experiment. The genus *Acinetobacter* was among the major taxonomic groups detected at t_2, t_3, and t_5.

3.2.3. Medium-level contamination experiments

Table 4 displays the microbial group frequency of larvae reared on wheat middlings inoculated with 5 log cfu *S. aureus* g^{-1} (Panel a). *Staphylococcus* was absent in larvae collected at t_2, whereas it was detected at the end of the experiment with 11.53%. The bacterial taxa *Moraxella*, *Anaerococcus*, and *Streptococcus* increased over time, reaching 44.34, 16.90, and 10.22% of the relative frequency at t_5. The genera *Bacillus*, *Acinetobacter*, *Exiguobacterium*, and *Pseudomonas* were dominant at t_2 with 42.68, 33.36, 13.25, and 10.07%, respectively, and greatly decreased over time.
Panel b of Table 4 shows the microbial group frequencies of wheat middlings and frass of larvae inoculated with 5 log cfu g^{-1} of *S. aureus*. *Staphylococcus* was detected at all the sampling times, with values comprised between 39.17% (t_1) and 1.54 (t_4). *Pseudomonas*, *Enterococcus*, and *Anaerococcus* registered an increase during time, with 23.70, 11.21, and 7.45% of the relative frequency at the end of the experiment. Instead, the genus *Pantoea* decreased from 66.67 (t_0) to 4.67% (t_5). As for *Peptoniphilus*, such microbial group displayed high frequencies at t_0, t_4, and t_5 (25.00, 31.67, and 10.61%, respectively).
The metataxonomic analysis results of control wheat middlings inoculated with 5 log cfu g^{-1} of *S. aureus* are reported in Table 4 (Panel c). *Staphylococcus* represented the main ASV at the beginning of the experiment with 54.00% of relative frequency, and showed stable values around 25% at t_3, t_4, and t_5. The microbial taxa *Acinetobacter*, *Enhydrobacter*, *Parvimonas*, and *Moraxella* increased up to 29.69, 15.89, 15.64 and 8.43%, respectively, at the end of the experiment. The genus *Streptococcus* was detected at all the sampling times, with values ranging from 1.22 (t_5) and 37.78% (t_1) of the relative frequency.

3.2.4. High-level contamination experiments

The results of the metataxonomic analysis of larvae reared on wheat middlings inoculated with 7 log cfu g^{-1} of *S. aureus* are reported in Table 5 (Panel a). *Staphylococcus* was slightly detected at t_2 with a relative frequency of 0.18%, whereas it resulted absent in the other sampling times. Although not detected from t_2 to t_4, the microbial taxa *Shinella*, *Enterobacter*, and *Clostridium* registered a substantial increase at the end of the experiment, with a relative frequency of 46.68, 35.99, and 12.22%, respectively. Conversely, the genera *Bacillus* and *Acinetobacter* displayed high frequencies only at t_0, with 53.58 and 29.53%, respectively. *Micrococcus* was the predominant ASV at t_2, reaching 99.81% of the relative frequency.
Regarding wheat middlings and frass of larvae rearing inoculated with 7 log cfu g^{-1} of *S. aureus*, data are reported in panel b of Table 5. *Staphylococcus* showed a decreasing trend over time, with a relative frequency of 41.92 and 0.88% at t_0 and t_5, respectively. *Enterococcus*, *Pseudomonas*, *Pantoea* increased during time, with values up to 26.34, 25.26, and 9.59% at the end of the experiment. As for *Streptococcus*, such genus was among the most representative groups of t_0 (19.87%) and t_1 (26.99%), whereas was absent at t_5. The taxa *Massilia* and *Sphingomonas* displayed a similar trend, as they were detected in most of the samples with a stable relative frequency of about 9 and 6%, respectively.
Panel c of Table 5 shows the microbial group frequencies of control wheat middlings inoculated with 7 log cfu g^{-1} of *S. aureus*. *Staphylococcus* was detected at all the sampling times, from 30.70 to 16.26% of the relative frequency at t_0 and t_5, respectively. *Finegoldia*, *Peptoniphilus*, and *Moraxella* represented the major ASVs at the end of the experiments, with 21.71, 18.10, and 17.40% at the end of the experiment. Instead, *Pseudomonas* was among the predominant taxa at t_1 (32.93%) and t_3 (42.49%), whereas it was slightly detected at t_4 (0.23%) and t_5 (0.57%). The genus *Streptococcus* displayed a stable trend over time, with values around 15% of the relative frequency in most of the sampling times.

4. Discussion

In foodstuffs, *S. aureus* represents a serious risk for the consumers due to the production of emetic and pyrogenic thermoresistant toxins (Fueyo, Mendoza, & Cruz Martín, 2005). The pathogen could reach the food production chain through naturally contaminated raw materials or via cross-contamination from the environment to the foodstuffs. Of note, warm-blooded animals, including humans can be natural carriers of *S. aureus* that is naturally harbored on the skin and hair (Grispoldi, Karama, Armani, Hadjicharalambous, & Cenci-Goga, 2021).
Regarding the edible insects food chain, Gorrens et al. (2021) recently published the results of a research dealing with the dynamics of *S. aureus* naturally occurring or artificially inoculated in substrates for black soldier fly larvae. Gorrens et al. (2021) highlighted that *S. aureus* inoculated in chicken feed used as substrate for black soldier fly larvae was not detected in the larvae and was reduced in the substrate after 6 days. The authors also suggested the need for further inoculation trials to investigate whether the observed reduction was

substrate dependent or not. It is noteworthy that, the study of Gorrens et al. (2021), although reporting encouraging results on the risk of *S. aureus* in the edible insects food chain, was carried out during a short-time period. Regarding this latter aspect, McGonigle, Purves, & Rolff (2016), who studied the intracellular survival of *S. aureus* during persistent infection in the insect *T. molitor*, evidenced how the current practice of studying short-term persistence of pathogens in insects could sometimes be misleading. Indeed, these authors successful recovered the pathogen 21 days after insect infection, thus demonstrating that long-term low-level persistence could represent a potential source of chronic infections.

In the present long-term study, the results of bacterial counts carried out on the inoculated wheat middlings, the frass, and the insect larvae showed a progressive reduction in the loads of *S. aureus* from t_1 to t_5 (56 days of rearing), that in frass and wheat middlings was depending on the inoculation level.

It is noteworthy that the growth of *S. aureus* is strongly influenced by the a_w of the substrate. As reported by Ewald & Notermans (1988) the minimum a*w* for *S. aureus* attest at 0.86; this feature could, at least partially, explain the low counts of *S. aureus* detected in the wheat middlings and frass that were characterized by aw values ranging from 0.6 (in the inoculated wheat middlings at t_0) to 0.5 in the frass at the end of rearing. As reported by Gorrens et al. (2021), although a low a_w represents a valid hurdle to reduce or inhibit bacterial growth, some pathogenic bacteria can survive the hostile environment and eventually multiply again when the water activity increases again; this feature could partly help to explain the variability of the viable counts detected in the present study at certain sampling times (e.g., frass at t5 – high-level contamination). Of note, despite the metabolic activity of larvae and the production of frass during rearing, no appreciable increase of a_w values was detected in the analyzed samples.

Regarding larvae, the results of the present study are in accordance with those reported by Gorrens et al. (2021) who suggested a reducing effect of the insect in *S. aureus* viability. Of note, the experiments of Gorrens et al. (2021) were carried out in black soldier fly rearing chain, hence, the comparison of data obtained in the presents study with those obtained by Gorrens et al. (2021) should be carried out taking into consideration the different physiological traits of the two insects.

Interestingly, the counts of *S. aureus* in the larvae fed the inoculated substrates were always below the detection limit (1 Log cfu g^{-1}) from t_2 until the end of rearing, irrespective of the initial inoculation level. These results are particularly encouraging since it is likely that the insect does not represent a favorable host for this insect and human pathogen. Notwithstanding, McGonigle et al. (2016), demonstrated that *S. aureus* was able to survive both inside and outside of *T. molitor* immune cells for up to 21 days post-infection. The same authors also revealed that some *T. molitor* individuals were able to achieve complete or near-complete clearance of the bacterial load during time (McGonigle et al., 2016).

As reported by Schelin et al. (2011), the presence of competitive microorganisms exerts a high impact on the growth rate and toxin formation of *S. aureus*. Hence, in order to obtain an overview of the microbial populations occurring in the experimental rearing trials, a metataxonomic analysis on cDNA synthesized from microbial RNA was carried out on wheat middlings used as feed, frass, insect eggs and larvae.

As for eggs, although viable counts revealed the absence of viable *S. aureus*, the metataxonomic analysis showed a high occurrence of *Staphylococcus epidermidis*. The occurrence of staphylococci in mealworms has already been observed since other species of *Staphylococcus* as *Staphylococcus warneri*, *Staphylococcus pasteuri*, *Staphylococcus kloosi*, and *Staphylococcus cohnii* were already detected in mealworms by Milanović et al. (2018). Of note, the presence of staphylococci in Coleoptera as buffalo worms and mealworms has also been reported by Grabowksi & Klein (2017), thus suggesting a high adaptation of this bacterial group to beetles.

Regarding the results of metataxonomic analysis carried out in all the samples, but eggs, sequencing results were unable to highlight the unequivocal presence of *S. aureus* in all the samples. This could represent a limitation of the study; notwithstanding, when the attribution of species was reached, *S. aureus* resulted in very low abundance, thus confirming the respective low viable counts. Moreover, it is likely that the high relative abundance of *Staphylococcus* detected in all samples originated from *S. epidermidis* contaminating the eggs.

Of note, the Baird-Parker Agar growth medium used in the present study for viable counting already showed by far a recovery capability of damaged staphylococci higher that other recovery media (Baird-Parker, & Davenport, 1965), with 97.5% of the *S. aureus* strains developing characteristic colonies (black, shiny colonies with white and clear zones) (Broeke, 1967). Interestingly, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* do not cultivate or do not show characteristic colonies on Baird-Parker Agar, thus further explaining the apparent discrepancy between the occurrence of *Staphylococcus* ASVs detected in samples by metataxonomic analysis and the results of viable counting.

Moreover, *S. aureus* and has been found to enter the viable but nonculturable (VBNC) state under some environmental stresses (e.g., low temperatures, dry aerosol, et.) (White, Nielsen, Møller Larsen, & Mette Madsen, 2020; Yan, Li, Meng, & Zhao, 2021); in such a physiological condition, *S. aureus* is still alive, but it can elude detection based on the use of conventional culture methods (Yan et al., 2021). Of note, the estimation of microorganisms in the VBNC state can be achievable using fluorescent microscopic techniques, immunological techniques, genetic-based techniques, ATP bioluminescence, and cytometry (Erkmen, 2022).
To explain the low levels of *S. aureus* cells in the larvae reared in the present study, many factors can be considered, including the immune system of the host, the interactions occurring between the inoculated microorganism and the microbiota in the gut of larvae (e.g., competitive exclusion by the endogenous larval microbiota), and insect species (e.g., antibacterial activity of the larvae) (Cesaro et al., 2022).
As reported by Dobson, Johnston, Vilcinskas, & Rolff (2012), the mealworm *T. molitor* exhibits a notable long-lasting stimulated humoral immune response to inoculation with live bacteria. Indeed, as reported by Haine, Moret, Siva-Jothy, & Rolff, 2008, *S. aureus* infection of *T. molitor* leads to fast initial killing of the pathogen, with a low level of persistent infection corresponding to enduring induced antimicrobial activity to defend the insect from chronic reinfection. Moreover, in many invertebrates, trans-generational immune priming gives immune protection to the offspring whose mothers have been exposed to the same bacterial pathogens (Dubuffet, Zanchi, Boutet, Moreau, Teixeira, & Moret, 2015). Interestingly, in *T. molitor*, the antibacterial activity found in the eggs is only active against Gram-positive bacteria (as *S. aureus*) with an insect-produced antibacterial peptide from the defensin family, namely tenecin 1, being directly active toward Gram-positive bacteria (Dubuffet et al. 2015).
Metataxonomic analyses showed the presence of many competing species in all the samples. It is noteworthy that *S. aureus* is a bacterium that advantages of the absence of competing species, whereas it is unable to compete for nutrients in the presence of commensal microorganisms (Parlet, Brown, & Horswill, 2019). As previously reported, in the present study, a high occurrence of *Staphylococcus* ASVs was detected among samples, with eggs showing high relative abundance of *S. epidermidis*. Interestingly, Parlet et al. (2019) reported that commensals *Staphylococcus* species belonging to the non-pathogenic coagulase-negative group, as *S. epidermidis*, *S. hominis*, *S. haemolyticus*, *S. capitis*, *S. lugdunensis*, and *S. warneri*, are able to effectively inhibit *S. aureus* colonization of animal tissues through the production of produce antibiotic-like compounds. Although this mechanism has been studied in mammal tissue, it could help to further explain the reduction in viable cells of *S. aureus* in the experimental rearing trials.
As suggested by Gorrens et al. (2021), despite the declining of *S. aureus* counts during rearing, the possible presence of enterotoxins produced by the pathogen in the rearing substrate or in the larvae could still represents a concern. In the present study, the absence of staphylococcal toxins in all the analyzed samples represents another encouraging result that increases the knowledge on the risks associated with *S. aureus* in the edible insects rearing chain. It is noteworthy that a_w, pH, and temperature represent significant factors affecting the probability of toxin production by *S. aureus* (Ding et al. 2016); moreover, as reported by Al-Nabulsi et al. (2020) the production of enterotoxins is not necessarily associated with staphylococcal growth, especially in food. Regarding a_w, minimum values comprised between 0.864 and 0.867 are required for the production of staphylococcal enterotoxins, hence, their absence in the analyzed wheat middlings or frass was almost expected, although the complexity and the novelty of the system under study justified the need for objective measurements. Furthermore, the production of toxins is strain dependent, and the most detected five toxins, namely enterotoxin A, B, C, D, and E, are encoded by specific genes such as *sea*, *seb*, *sec*, *sed*, and *see,* respectively (Omwenga et al., 2019). Hence, in order to completely exclude the risk of toxin formation the detection of the abovementioned genes is also suggested.
In the present study, no unusual mortality of larvae was observed throughout the entire rearing period, thus suggesting that the inoculation of *S. aureus* in the rearing substrates did not reduce the viability of mealworms, irrespective of the initial contamination level.

Conclusions

CRediT authorship contribution statement

Cristiana Cesaro: Investigation, Formal analysis, Writing - Original Draft. **Cinzia Mannozzi:** Investigation, Formal analysis. **Adolfo Lepre:** Investigation, Formal analysis. **Ilario Ferrocino**: Investigation, Formal analysis, Writing - Original Draft. **Lorenzo Corsi:** Formal analysis. **Irene Franciosa:** Formal analysis.

Annalisa Petruzzelli: Formal analysis. **David Savelli**: Formal analysis. **Luca Belleggia**: Investigation. **Vesna Milanović:** Investigation, Resources. **Federica Cardinali:** Investigation, Resources. **Cristiana Garofalo:** Investigation, Resources. **Luca Cocolin**: Writing - original draft. **Lucia Aquilanti:** Writing - Original Draft, Resources. **Sara Ruschioni**: Writing - Original Draft, Resources. **Nunzio Isidoro:** Writing - Original Draft, Resources. **Paola Riolo:** Writing - Original Draft, Supervision, Resources. **Andrea Osimani:** Conceptualization, Writing - Review & Editing, Supervision, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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