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Fate of *Escherichia coli* artificially inoculated in *Tenebrio molitor* L. larvae rearing chain for human consumption

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Abstract

The edible insect food chain represents a relative novel food-producing system, hence, associated biological risks has still to be exhaustively evaluated. In the present study the dynamics of *Escherichia coli* during the whole living period of *Tenebrio molitor* larvae (from eggs to pupae) were studied. To this end, a rearing substrate consisting of organic wheat middlings was spiked with *E. coli* cells at two initial contamination levels 1 log cfu g^{-1} (low) and 6 log cfu g^{-1} (high). Microbial viable counting coupled with metataxonomic analyses were useful to assess: i) the persistence and growth of *E. coli* in the rearing substrate (wheat middlings); ii) the colonization and growth of *E. coli* in the gut of the insect larvae; and iii) the occurrence and load of *E. coli* in the frass (excrement from larvae mixed with substrate residues). The results highlighted a very limited persistence of the pathogen in all the analyzed samples. In more detail, the results suggested that when *E. coli* was present at very low levels in the eggs of the insect, the pathogen was not able to reach concerning levels in the larvae. Moreover, when *E. coli* was present in the wheat middlings used for rearing, the environmental conditions of the substrate (low a_w values) were not favorable for its survival and multiplication, irrespective of the presence of the larvae and their frass. Surprisingly, at the conditions applied in the present study, the larvae fed wheat middlings contaminated with *E. coli* seemed to be inhospitable or even hostile environment for microbial survival or multiplication. To explain the reduction of *E. coli* cells in the larvae reared in the present study, many factors can be considered, among these: the immune response of the host, microbial composition and interactions established in the gut of larvae, and insect species. Of note, part of the major fraction of the microbiota of larvae at the end of rearing was represented by *Lactococcus*, thus suggesting a possible effect of this lactic acid bacterium in *E. coli* survival. Further research is needed to better clarify the interactions between *E. coli* and the insect gut, as well as the interactions established among the target microorganism and those naturally harbored by the insect gut.

Keywords: edible insects; challenge test; metataxonomic analysis; bacterial infection; risk assessment.

1. Introduction

Edible insects farming for food production represents a frontier agriculture for turning a linear food economy into a circular one (Verner et al., 2021). Indeed, edible insects are easy to be reared on several substrates, including agriculture or food industry by-products (e.g., wheat middlings, olive pomace, etc.) (Ruschioni et al., 2020; Osimani et al. 2018). Moreover, edible insects are rich in protein, minerals, and fat, thus representing a high-value ingredient for food preparations (Roncolini et al., 2020).

Conscious of this potential, the European Union (EU) has pushed the exploitation of edible insects since 2015. Indeed, in that year, the European Food Safety Authority (EFSA) published the first scientific opinion on a risk profile related to the production and consumption of insects as food and feed, and suggested a list of edible insects to be used for these purposes (EFSA, 2015). Subsequently, Regulation (EU) 2015/2283 on novel foods established the procedure for obtaining the permission of commercialization of foods containing insects and their parts (Regulation (EU) 2015/2283). Among the edible insects suggested by the EFSA as suitable for human consumption, *Tenebrio molitor* L. undoubtedly represents one of the most promising species. Of note, European Commission Implementing Regulation (EU) 2021/882 recently authorized the placing on the market of dried *T. molitor* larva as a novel food (Commission Implementing Regulation (EU) 2021/882), thus opening new market perspectives to European food industries.

From the time edible insects attracted the attention of the EU, and according to the EFSA recommendations (EFSA, 2015), it was clear that a proper risk assessment should have been performed in order to assess potential safety risks for the consumers. Therefore, to increase the knowledge on the safety and exploitability of edible insects, the research sector began to move in parallel. It is now clear that edible insects can be vectors of physical, chemical, and biological hazards that must properly be evaluated and managed during rearing and processing of insects (Garofalo et al. 2019).

Among safety hazards carried by edible insects, microorganisms represent one of the major concerns for public health. Of note, edible insects can be infected by microorganisms through a direct contamination of the eggs from the mother to the offspring during egg-laying (Osimani et al., 2021). Moreover, microorganisms can also contaminate the external cuticle of insects as well as their gut through the ingestion of contaminated feed (Garofalo et al. 2019). Among the most detected microorganisms, those belonging to the Enterobacteriaceae

family surely represent the major fraction (Garofalo et al., 2019). This bacterial family includes saprophytic microorganism together with those with potential pathogenic effect on humans, including *Salmonella* spp., *Klebsiella* spp. *Erwinia* spp., *Enterobacter* spp., and *Escherichia coli*.

E. coli is a Gram-negative mesophilic bacterium naturally occurring as commensal microorganism in animal or human gut. A major part of *E. coli* strains does not present a health risk; however, some strains have the ability to attack organs or systems of the human body causing diseases (Aijuka & Buys, 2019). In more detail, pathogenic *E. coli* can be the causative agents of gastrointestinal or extraintestinal disease, this latter including urinary infections, septicemia, and meningitis (Aijuka & Buys, 2019). Pathotypes usually related with human disease include, adherently invasive *E. coli* (AIEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and shigatoxin-producing *E. coli* (STEC) (Croxen et al., 2013).

E. coli is included in the major zoonoses that are constantly monitored in humans, food, animals, and feed by the EFSA and the European Centre for Disease Prevention and Control (ECDC) (EFSA & ECDC, 2020).

E. coli is not a heat-resistant microorganism that is inactivated by thermal treatments (e.g., pasteurization), however, this foodborne pathogen can reach the human gut through the ingestion of contaminated food that has not been properly processed (e.g., improper heat treatment). Moreover, *E. coli* can contaminate foods after processing, due to cross-contamination between ready-to-eat food and raw materials or tools that entered in contact with animal feces. In food, the presence of *E. coli* is commonly used to assess enteric contamination and, hence, the hygiene of production (Petruzzelli et al., 2016).

Although many studies on the occurrence of microorganisms in edible insects have been published in the available scientific literature, to the authors' knowledge, only a limited number of published studies deal with the dynamics of specific pathogens in the edible insect food chain (Belleggia et al., 2020; De Smet et al., 2021; Erickson, Islam, Sheppard, Liao, & Doyle, 2004; Gorrens et al., 2021; Liu, Tomberlin, Brady, Sanford, & Yu, 2008; Mancini et al., 2019; Wynants et al., 2019).

In the present study the dynamics of *E. coli* during the whole living period of *T. molitor* larvae (from eggs to pupae) were studied. To this end, a rearing substrate, consisting of organic wheat middlings, was spiked with *E. coli* cells at two initial contamination levels (low and high). Microbial viable counting coupled with metataxonomic analyses were performed to evaluate: i) the persistence and growth of *E. coli* in the rearing

substrate; ii) the colonization and growth of *E. coli* in the gut of the insect; and iii) the occurrence and load of *E. coli* in the frass (excrement from larvae mixed with substrate residues).

2. Materials and methods

2.1. Experimental design

In order to contaminate *T. molitor* larvae with the test microorganism, organic wheat middlings used as rearing substrate was spiked with a multi-strain cocktail of *E. coli* cells at two different contamination levels, namely 1 (low-level) and 6 (high-level) log colony-forming units (cfu) per gram.

For each contamination level, three replicates were set up (F_1, F_2, F_3). In more detail, F_1, F_2, and F_3 trays contained organic wheat middlings spiked with *E. coli* at the different contamination levels and *T. molitor* eggs, whereas a control tray (F_C) did not contain eggs, since it was used to evaluate *E. coli* behaviour in the sole rearing substrate during time.

Moreover, three control trays (C_1, C_2, and C_3), containing *T. molitor* eggs and wheat middlings, were set up with no *E. coli* inoculum to monitor the natural insect development from eggs to pupae during time.

Prior to start the experiment, wheat middlings and 1 gram of eggs (about 1800 eggs) were tested for the absence of *E. coli* by plate counting and metataxonomic analysis (as described in the following paragraphs).

Samples of eggs and wheat middlings were collected at t0, whereas samples of larvae and frass of F_1, F_2, F_3, and F_C as well as C_1, C_2, and C_3 were collected at regular intervals (every 14 days) from the day of *E. coli* inoculum (t_0). Due to the very small size of larvae at the beginning of rearing, their sampling started at t_2 (28 days). The trial was stopped as soon as larvae turned into pupae (56 days) (Figure 1).

2.2. Inoculum preparation

Two different *E. coli* strains were used. In more detail, *E. coli* ATCC 25922 was purchased from the American Type Culture Collection (Manassas, Virginia, United States); moreover, the second *E. coli* strain used in the

present study was previously isolated from raw meat and was kindly provided by Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Perugia (Italy).

Both strains were separately sub-cultured twice in Brain Heart Infusion (BHI) broth (Sigma, Milan, Italy) at 37 °C for 24 hours. The biomasses of the last sub-cultures were centrifugated with a Rotofix 32A centrifuge (Hettich, Tuttlingen, Germany) at 4,000 rpm for 10 minutes. The resulting supernatants were removed, and cell pellets were resuspended in physiological water solution (0.85% NaCl). The absorbance of each suspension was measured at 600 nm using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and cell viability was evaluated through viable counting on BHI agar (Sigma). Finally, for each strain, two bacterial suspensions were set up by diluting the initial suspensions with physiological solution to reach the contamination levels required: 1 and 6 log cfu g^{-1}.

2.3 E. coli inoculation

Initially, 680 g of organic wheat middlings were placed in each tray and utilized as rearing substrates. 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). Aliquots (3.4 mL for each strain) of *E. coli* suspensions were spiked as follows: four trays at 1 log cfu g^{-1} contamination level and four trays at 6 log cfu g^{-1} contamination level. Each inoculum was distributed in the rearing substrates spreading 170 droplets (20 µL each) with a semi-automatic pipette. After inoculation, the rearing substrates were thoroughly mixed using a sterile spoon. Five grams of the spiked rearing substrates were randomly collected from all the replicates to verify the load of inoculated *E. coli* (t_0).

2.4 Mealworm larvae rearing and sampling

Approximately four kilograms of mealworms were purchased from INEF - Insect Novel Ecologic Food factory (Padova, Italy) to set up the mother colony that was fed organic wheat middlings in a climate-controlled chamber maintained at 28 ± 0.5 °C and at a relative humidity of 60 ± 0.5%. Once larvae were turned into pupae, they were collected, sexed, and separated according to their sex until reaching the adult stage. Finally,

300 adults (sex ratio 1:1) were placed in plastic trays containing organic wheat middlings and filter paper discs to favour the egg adhesion. The eggs (1,000 for each selected tray) were placed into the trays containing spiked wheat middling (F_1, F_2, and F_3), except for F_C boxes, and the three control trays containing uninoculated wheat middling (C_1, C_2, C_3). All the boxes were kept into the climate-controlled chamber as described above. Twice a week, ten grams of previously washed, peeled, and sliced carrots were added to each rearing experiment as a source of water for larvae (Osimani et al., 2018). Carrots were also tested for the absence of *E. coli*.

Aliquots of mealworm larvae and frass were collected from each batch every 14 days. Prior to analysis, larvae were frozen at -20 °C for 40 minutes, washed with a 70% ethanol solution in sterile deionized water, rinsed twice in sterile deionized water (Wynants et al., 2019), and then homogenized in a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy).

Frass samples were collected in sterile bags for microbiological viable counts (5 g) and for water activity (a_w) measurements (5 g).

The manipulation of rearing substrates, eggs, larvae, and frass was carried out using sterile gloves and sterile tools in order to avoid cross-contamination between samples and the environment.

2.5. Water activity measurement

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 were collected starting from t_2, weighted, and added with suitable amount of sterile peptone water to reach 1:10 ratio for microbiological viable counts. The sample homogenization was carried out for 1 min at 260 rpm using a Stomacher apparatus (International PBI, Milan, Italy). The resulting suspensions were ten-fold diluted in sterile peptone water, plated on Chromogenic Coliform Agar (CCA) medium (VWR, Leuven, Belgium) and incubated at 37° C for 24 hours to enumerate *E.*

coli. For each analysed sample, the results were expressed as the log cfu g^{-1} and reported as mean of triplicate experiments ± standard deviation.

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 RNA later 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).

The microbial RNA was extracted through 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 procaryotic 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). The cDNA synthesis was performed using the SensiFAST cDNA Synthesis Kit (Bioline, London, UK) in accordance with manufacturer's instructions.

2.8. Metataxonomic analysis

Samples were analyzed by a metataxonomic approach in order to study the microbiota composition and to monitor the presence of the inoculated *E. coli* during insect rearing. cDNA was used to amplify the V3-V4 region of the 16S rRNA gene using primers and protocol by Klindworth et al. (2013). Purification of amplicons, tagging, and pooling were performed following Illumina guidelines. The MiSeq platform (Illumina) was used to generated 250-bp paired-end reads and the obtained raw files (*.fastq*) were imported in QIIME 2 software (Bolyen et al., 2019). Primers were removed by cutadapter and sequences were denoised by DADA2 algorithms in QIIME 2, in order to obtain Amplicon Sequence Variants (ASVs) (Callahan et al., 2016).

Taxonomy classification was performed using the *SILVA* database v.13.8 by the QIIME 2 feature-classifier script. The ASVs with less than 0.5% in at least two samples were excluded. Alpha-diversity index and rarefaction curves were calculated using the qiime diversity script of QIIME2

Sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (PRJNA802576).

2.9. Statistical analysis

After first checking for conformance to a normal distribution, the Tukey-Kramer's Honest Significant Difference (HSD) test (level of significance 0.05) was used to evaluate differences of viable counts and physico-chemical parameters within samples by one-way analysis of variance (ANOVA). Tests were carried out using the software JMP Version 11.0.0 (SAS Institute Inc., Cary, NC).

ASVs 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) and visualized by using the corrplot package of R.

3. Results

3.1. Bacterial counts

Viable counts carried out in all the experimental trays are reported in Table 1. The counts of the rearing substrates sampled at t_0 indicated that the desired contamination levels (1 and 6 log cfu g^{-1}) were achieved in all the trays. After 14 days (t_1), a statistically significant reduction of *E. coli* cells was observed in all the experiments. In more detail, at t_1 *E. coli* levels were lower than 1 log cfu g^{-1} in wheat middlings spiked with 1 log cfu g^{-1}, whereas, at the same sampling time, in the wheat middlings spiked with 6 log cfu g^{-1}, *E. coli* levels rapidly decreased reaching about 4 Logcfu g^{-1}. A further statistically significant reduction in the *E. coli* loads was highlighted at t_2. In fact, in the wheat middlings spiked with high-level contamination, the counts reached

about 3 log cfu g^{-1}. Finally, starting from t_3, in all the samples, *E. coli* loads were always lower than 1 log cfu g^{-1}.

As regards larvae samples, the presence of the target microorganism was never detected at all sampling times starting from t_2.

A_w values detected in all the analyzed samples are reported in Table 1. In more detail, the a_w values recorded in the present study were comprised between 0.4057 and 0.6124. A progressive increase in the a_w values was observed during the experimental timespan, with final values attesting around 0.57 in all the wheat-based substrates.

3.2. Metataxonomic analysis

Alpha rarefaction curves obtained shown to reach a plateau, indicating that most biodiversity was captured by the applied analysis (see Fig. S1) moreover a satisfactory coverage was obtained (>98%) Table S1

3.2.1. Control experiments

In the analyzed *T. molitor* eggs, *Escherichia* was present at very low frequency (0.14%) (Figure 2). Eggs microbiota was dominated by *Lactococcus* (about 65% of the relative frequency) followed by *Lactobacillus* and *Sphingomonas* (about 2% each). *Massilia*, *Methylobacterium* and *Paenibacillus* were detected as minor ASVs (1.9 – 1.3%) (Supplementary Table 1).

As for larvae reared on uninoculated wheat middlings (Figure 3, panel a), a decrease in the relative frequency of *Escherichia* was observed from t_2 (0.17%) to t_4 (absent). Moreover, a decrease of most of the detected ASVs was observed over time. In more detail, *Corynebacterium, Curvibacter, Lactococcus, Methylobacterium, Micrococcus, Ralstonia, Sphingomonas,* and *Staphylococcus* decreased from more than 3% to less than 1% of the relative frequency. At the end of the experimental trial, *Enterococcus* represented the dominant ASV (Supplementary Table 2).

Regarding frass of larvae reared on uninoculated wheat middlings (Figure 3, panel b), *Escherichia* was not detected in any of the analyzed samples. *Bacillus* and *Enterococcus* were not detected from t_0 to t_2, whereas an increase in their frequency was observed at t_3 and t_4 up to 20% and 38%, respectively. In addition, a decrease

in the relative frequencies of *Curtobacterium*, *Pedobacter*, *Paenibacillus* and *Xylophilus* was observed from t_0 to t_4 (Supplementary Table 3).

3.2.2. Low-level contamination experiments

In larvae samples fed wheat middlings spiked with 1 log cfu g^{-1} (Figure 4, panel a), *Escherichia* (together with *Bacillus*) showed a slight increase in the relative frequency at t_4 (Supplementary Table 4), whereas a decrease in the relative frequency of *Sphingomonas* and *Methylobacterium* was observed at the same sampling time (t_4) (Supplementary Table 4). Co-occurrence analyses (Figure 5) showed that in the larvae, the presence of *Escherichia* was negatively correlated with *Aureimonas, Brevundimonas, Chryseobacterium, Massilia, Paenibacillus, Paracoccus, Pedobacter,* and *Pigmentiphaga*. Whereas the presence of *Bacillus* co-occurred with *Escherichia*.

As for frass from rearing of larvae (Figure 4, panel b), the bacterium was detected at low frequency (0.32%) only at t_0. An increase of *Bacillus*, *Lactobacillus*, and *Lactococcus* was observed at t_4, whereas the relative frequency of *Chryseobacterium, Curtobacterium, Hymenobacter, Massilia, Rhizobium, Roseomonas* and *Saccharibacillus* gradually decreased from t_0 to the end of the trial (Supplementary Table 5).

In the wheat middlings samples inoculated with 1 log cfu g^{-1} of *E. coli* and used as control (without larvae) (Figure 4, panel c), *Escherichia* was present at low frequency (0.5 %) only at t_0. In control wheat middlings samples the predominant ASVs were *Chryseobacterium, Curtobacterium, Massilia, Methylobacterium, Paenibacillus, Pedobacter, Rhizobium,* and *Sphingomonas*. *Bradyrhizobium* and *Corynebacterium* were present at high frequency at t_0 (about 5%) but they were absent at t_1 (Supplementary Table 6).

3.2.3. High-level contamination experiments

Regarding larvae samples inoculated with 6 log cfu g^{-1} (Figure 6, panel a), a decrease in the relative frequency of *Escherichia* was observed over the time from 2.1% at t_2 to 0% at t_4. In the analyzed samples *Lactococcus* represented the dominant ASV at t_4 (97% of the relative frequency). Moreover, a decrease in the relative frequency of several ASVs (*Bacillus, Bradyrhizobium, Curvibacter, Chryseobacterium, Ralstonia, Sphingomonas* and *Methylobacterium*) was observed over time (Supplementary Table 7). Co-occurrence

analyses of data collected from larvae (Figure 7, panel a) showed that that the presence of *Lactococcus* co-excluded *Escherichia*.

As for frass from rearing of larvae (Figure 6, panel b), a decrease in the relative abundance of *Escherichia* from 14.2% at t_0 to 3.6% at t_4 was observed. *Paenibacillus* and *Sphingomonas* were the ASVs that showed the highest frequency (more than 10%), followed by *Curtobacterium, Massilia, Methylobacterium*, and *Rhizobium*. Finally, an increase in the relative frequency of *Bacillus* from 0.9% to 36.5% was observed at t_4 (Supplementary Table 8). Figure 7 (panel b) shows the positive correlation between the presence of *Escherichia* and *Rhodococcus*.

In the wheat middlings samples used as control (without larvae) inoculated with 6 log cfu g^{-1} of *E. coli* (Figure 6, panel c), *Escherichia* was the dominant genus at t_0 (44.7%), whereas its frequency gradually decreased over time to 3% at t_4. An increase in the relative abundance of *Chryseobacterium, Paenibacillus, Pedobacter, Roseomonas*, and *Sphingomonas* was observed from t_0 to t_4 (Supplementary Table 9). The co-occurrence analyses of data collected from wheat middlings without larvae (Figure 7, panel c), highlighted a negative interaction between *Escherichia* and *Chryseobacterium, Sanguibacter,* and *Sphingomonas*. Whereas the presence of *Hymenobacter* was positively correlated to the presence of *Escherichia*.

4. Discussion

Edible insects represent a peculiar novel source of high-value protein, and their exploitation poses safety concerns for the consumers that have to be carefully investigated. Microbiological challenge tests offer the opportunity to explore the behavior of specific microorganisms in this novel food matrix.

The present study is based on the hypothesis that, in the case that *E. coli* contaminates the feed of *T. molitor*, the microorganism could infect the larvae, and progressively multiply in their gut. Hence, the resulting frass could act as a further inoculum of the larvae, thus leading to a further increase in the total number of viable *E. coli* cells in both the insect gut and frass during rearing.

The analysis of RNA could be applied to evaluate the living microbial communities in food matrices (Garofalo et al., 2017). Hence, in the present study, the combination of viable counting with metataxonomic analysis,

performed on the cDNA synthesized from microbial RNA, provided a sound overview of the fate of *E. coli* inoculated in wheat middlings used as feed and, subsequently, in the larvae gut.

In the present study, the results of viable counts at t_0 attested that, in the rearing substrates, the two desired inoculum levels (1 and 6 log cfu g^{-1}) of *E. coli* were reached.

Regarding a_w monitored in the wheat-based substrates and frass, the slight differences of a_w measured in control or spiked wheat middlings at t_0 could likely be related to the addition of the aqueous suspensions of the target microorganism. Indeed, the lowest a_w average value was measured in the uninoculated control wheat middlings. Interestingly, a_w values were quite similar in all the rearing trials from t_1 to t_4, irrespective of the inoculum level. Similar a_w values were also detected in trays containing the sole wheat middlings spiked with *E. coli* (without larvae). Such findings suggest that larvae metabolism (and feces) did not affect water content of the rearing substrate (or frass) (from t_1 to t_4), moreover, it is likely that the recorded a_w reached the equilibrium with the environmental moisture, being this latter constant during all the experiment.

As reported by Michael et al. (2022), although *E. coli* outbreaks are generally related to animal-based food products, low-a_w foods (e.g., wheat flours or wheat by-products) have also been involved in some outbreaks (CDC, 2019). Interestingly, although *E. coli* is not able to multiply in the dry conditions of wheat flour (or wheat dry by-products), the microorganism can survive in the dry food matrix and grow as soon as the environmental conditions become favorable again (Eglezos, 2010). In the present study, viable counts of *E. coli* showed a progressive reduction in wheat middlings used as rearing substrate as well as in frass or wheat middlings used as control, irrespective of the inoculum level. The results of viable counting were confirmed by metataxonomic analyses that showed a parallel reduction in the relative abundances of the test microorganism. It is known that the optimal a_w value for *E. coli* growth ranges from 0.95 to 0.99 (Juneja et al., 2014), hence the lack in *E. coli* growth in the dry substrates (wheat middlings or frass), showing maximum a_w values around 0.57, was partly expected. Notwithstanding, further research is needed to evaluate the viability of *E. coli* inoculated on wet feed (e.g., carrots) as well as its eventual transmission to *T. molitor* larvae.

In the present study, viable counts of *E. coli* performed on the eggs of *T. molitor* showed values below the detection limit, notwithstanding, the metataxonomic analyses showed the presence of the target microorganism at a very low relative abundance. This result can be explained by the inevitable contamination of eggs from the microbiota of the mother during deposition (Vilcinskas, 2021). Of note, in the present study, eggs were not

sterilized in order to avoid dysbiosis of the larvae possibly affecting the gut equilibrium of the insects and, thus, misrepresenting a real situation.

It is noteworthy that insect gut could represent a favorable environment rich in water and nutrients for microbial growth (Muñoz-Benavent, Pérez-Cobas, García-Ferris, Moya, & Latorre, A., 2021). Hence, the reduction in the loads of *E. coli* in the larvae gut was quite unexpected. Metataxonomic analyses confirmed the reduction in viable *E. coli* cells in the larvae gut. The data collected in the present study were not in accordance with those collected by De Smet et al. (2021) in a similar study. Indeed, De Smet et al. (2021) found an increase over time of artificially inoculated *Salmonella* strains in both feed and *Hermetia illucens* larvae fed grinded chicken starter feed.

To explain the reduction of *E. coli* cells in the larvae reared in the present study, many factors can be considered, among these: the immune response of the host, microbial composition and microbial interactions established in the gut, and insect species.

It is noteworthy that in the insect gut complex and species-specific interactions between the host and the microbiota usually occur (Lemoine, Engl, & Kaltenpoth, 2020). As an example, microbial symbionts can contribute to the host's nutrition, digestion, and defense, being also able to affect the host's response toward abiotic stressors (Lemoine et al., 2020). Moreover, as recently reported by Ali Mohammadie Kojour et al. (2022), insects compensate a lack in adaptative immunity by a robust innate immunity exerted by cellular immune response, that includes phagocytosis, encapsulation, and nodulation. In addition, as reported by Vilcinskas (2021), parents can prepare offspring for defense against pathogens through a phenomenon known as specific transgenerational immune priming, thus maintaining the host immune system in a combat-ready state. Interestingly, Moret (2006) reported an inherited protection of *T. molitor* (until the F2 generation) against *E. coli* after immune priming of the insect parents with the lipopolysaccharide of the microorganism. A similar immune priming has been reported by Roth et al. (2010) for the coleoptera *Tribolium castaneum*. In the present study, it is conceivable that the very low amount of *E. coli* originally occurring in the eggs did not directly affect the results of the challenge tests. Moreover, it is likely that such low *E. coli* levels in the eggs were not able to multiply due to a possible priming of the offspring by the parents, thus leading to a subsequent resistance of the insect to the pathogen infection (Ali Mohammadie Kojour et al., 2022). Of note, insect transgenerational

immune priming could be used as preventive action to sustain resistance of the insect to microbial infections (Grau et al., 2017).

It is noteworthy that a humoral immune response can also be activated in insects through antimicrobial peptides produced in the fat body and secreted into the hemolymph. Interestingly, many target genes for antibacterial peptides active against *E. coli* have been discovered in *T. molitor* (Ali Mohammadie Kojour et al., 2022).

The metataxonomic analyses provided useful data to be compared with viable counts, confirming, on the one hand, the results of counts of *E. coli* in all the analyzed samples and, on the other, suggesting possible explanations to the reduction of the inoculated target microorganism.

Interestingly, a major fraction of the microbiota of eggs was represented by *Lactococcus*. The presence of *Lactococcus* suggests a massive contamination of eggs with this lactic acid bacteria by the mother. To the authors' knowledge, no studies have previously investigated the microbiota of *T. molitor* eggs, hence, further comparison of data is difficult to be performed. Of note, *Lactococcus* has already been detected in larvae of *T. molitor* reared on wheat middlings (Osimani et al., 2018), thus suggesting a strong adaptation of such microbial genus to the gut of *T. molitor*.

Interestingly, in *T. molitor* larvae fed wheat middlings with low-level contamination, the co-occurrence of *Escherichia* and *Bacillus* was observed. The presence of *Bacillus* spp. and other spore forming bacilli has already been described in edible insects by many authors (Fasolato et al., 2018; Garofalo et al., 2019; Osimani & Aquilanti, 2021). Among the most detected species, the presence of *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus cytotoxicus*, *Bacillus firmus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus simplex*, and *Bacillus sonorensis* has already been reported in edible insects (Garofalo et al., 2019). As reported by Xiang et al. (2021), among *Bacillus* species, *Bacillus subtilis* showed antibacterial properties, due to the production of bacteriocins (antimicrobial peptides synthesized by microorganisms), against *E. coli*. Moreover, as reported by Ekim, Calik, Ceylan, & Saçaklı (2020), *Paenibacillus* showed probiotic activity in chicken, being able reduce the number of *E. coli* in the cecum. It is likely that the presence of *Bacillus* spp. in the analyzed larvae samples could have contributed to maintain at low level the relative frequency, as well as viable counts, of *E. coli* in the larvae gut. Similarly, in *T. molitor* larvae fed high-contamination level wheat middlings, high relative abundance of *Paenibacillus* spp. were detected, with concurrent reduction, down to zero, of the relative frequency of *E. coli* at t_4. Hence, although the abovementioned activities of *Bacillus* and *Paenibacillus* have

still to be verified in *T. molitor*'s gut, they might be taken into consideration to partly explain the reduction of *E. coli* in the larvae under investigation.

In the present study, a part of the major fraction of the microbiota of larvae at the end of rearing (t_4) was represented by *Lactococcus* (likely deriving from eggs), occurring at 12% and 97% of the relative abundance in the larvae fed wheat middlings inoculated with 1 or 6 log cfu g^{-1} *E. coli* cells, respectively. Of note, bacteriocins produced by *Lactococcus* already proved to be effective in counteracting the viability of some foodborne pathogens, including *E. coli* (Bragason et al., 2020). Such evidence suggests that, in the insects' gut, *Lactococcus* could have produced antimicrobial compounds able to inhibit or even kill the inoculated target microorganism. Although this hypothesis has still to be confirmed, it is noteworthy that, in the analyzed *T. molitor* larvae fed high-level contamination wheat meddlings at the lowest relative abundance of *E. coli* (absence) corresponded the highest relative abundance of *Lactococcus* (97%) at t_4. Similarly, in the larvae fed low-level contamination wheat middlings, the reduction of *E. coli* at t_4 corresponded to the highest relative abundance of *Lactococcus* (12%). In this latter case, the relative abundance of *Lactococcus* was lower than that detected in the high-level contamination trials, thus explaining the persistence, although at very low relative abundance, of *E. coli* at t_4.

Conclusions

The edible insect food chain represents a relative novel food-producing system, hence, associated biological risks have still to be exhaustively evaluated. In this study, the fate of *E. coli* artificially inoculated in *T. molitor* larvae food chain has been assessed. Considering safety aspects, the encouraging results highlighted a very limited persistence of the pathogen in all the analyzed samples. In more detail, the results suggested that, when *E. coli* is present at very low levels in the eggs of the insect, the pathogen is likely not able to reach concerning levels in the larvae. Moreover, when *E. coli* is present in the wheat middlings used for rearing, the environmental conditions of the substrate (low a_w values) are not favorable for its survival and multiplication, irrespective of the presence of the larvae and their frass. Surprisingly, the gut of larvae fed wheat middlings contaminated with *E. coli* seems to be inhospitable or even hostile environment for microbial survival or

multiplication. Notwithstanding, good manufacturing practices as well as preventive actions aimed at minimizing the risk for the consumer have to be implemented in any case.

Further research is needed to better clarify the interactions between *E. coli* and the insect gut, as well as the interactions established among the target microorganism and those naturally harbored by the insect gut.

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. **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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FIGURE CAPTIONS

Fig. 1. Experimental design

t0, day of inoculation; t1, day 14; t2, day 28; t3 day 42; t4, day 56

F, biological replicates of frass

F_C, biological replicates of inoculated control wheat middlings

L, biological replicates of larvae

C, uninoculated control frass

L_C, biological replicates of larvae reared on uninoculated control wheat middlings

Fig. 2. Incidence (%) of the bacterial taxonomic groups in eggs detected by sequencing.

The incidence of *Escherichia* is indicated with yellow colour. The complete list of bacterial taxonomic groups detected in the sample is reported in Supplementary Table 1

Fig. 3. Incidence (%) of the bacterial taxonomic groups in larvae reared on uninoculated wheat middlings (panel a, Supplementary Table 2) and in wheat middlings (t_0) and frass (from t_1 to t_4) of larvae reared on uninoculated wheat middlings (panel b, Supplementary Table 3), as detected by sequencing.

Samples are labelled according to time (t0, day of inoculation; t1, day 14; t2, day 28; t3 day 42; t4, day 56). The incidence of *Escherichia* is indicated with yellow colour. The complete list of bacterial taxonomic groups detected in the sample is reported in Supplementary Tables 2 and 3

Fig. 4. Incidence (%) of the bacterial taxonomic groups in larvae fed wheat middlings inoculated with 1 log cfu g^{-1} of *Escherichia* (panel a, Supplementary Table 4), wheat middlings (t_0) and frass (from t_1 to t_4) from larvae fed wheat middlings inoculated with 1 log cfu g^{-1} of *Escherichia* (panel b, Supplementary Table 5), and

wheat middlings inoculated with 1 log cfu g^{-1} of *Escherichia* (panel c, Supplementary Table 6) without larvae, as detected by sequencing.

Samples are labelled according to time (t0, day of inoculation; t1, day 14; t2, day 28; t3 day 42; t4, day 56). The incidence of *Escherichia* is indicated with yellow colour. The complete list of bacterial taxonomic groups detected in the samples is reported in Supplementary Tables 4, 5, and 6.

Fig. 5. Significant co-occurrence and co-exclusion relationships between bacterial amplicon sequence variants (ASVs) in larvae reared on wheat middlings inoculated with 1 log cfu g^{-1}.

Spearman's rank correlation matrix of ASVs with >0.5% abundance in at least 2 samples. Strong correlations are indicated with large squares, whereas weak correlations are indicated with small squares. The color of the scale bar denotes the nature of the correlation with 1 indicating a perfectly positive correlation (dark blue) and 1 indicating a perfectly negative correlation (dark red). Only significant correlations ($P < 0.05$) are shown.

Fig. 6. Incidence (%) of the bacterial taxonomic groups in larvae fed wheat middlings inoculated with 6 log cfu g^{-1} of *Escherichia* (panel a, Supplementary Table 7), wheat middlings (t_0) and frass (from t_1 to t_4) from larvae fed wheat middlings inoculated with 6 log cfu g^{-1} of *Escherichia* (panel b, Supplementary Table 8), and in wheat middlings inoculated with 6 log cfu g^{-1} of *Escherichia* (panel c, Supplementay Table 9) without larvae, as detected by sequencing.

Samples are labelled according to time (t0, day of inoculation; t1, day 14; t2, day 28; t3 day 42; t4, day 56). The incidence of Escherichia is indicated with yellow colour. The complete list of bacterial taxonomic groups detected in the samples is reported in Supplementary Tables 7, 8, and 9.

Fig. 7. Significant co-occurrence and co-exclusion relationships between bacterial amplicon sequence variants (ASVs) in larvae reared on wheat middlings inoculated with 6 log cfu g^{-1} of *Escherichia* (panel a); in frass

from larvae reared on wheat middlings inoculated with 6 log cfu g^{-1} of *Escherichia* (panel b); and in wheat middlings inoculated with 6 log cfu g^{-1} of *Escherichia* (panel c) without larvae, as detected by sequencing.

Spearman's rank correlation matrix of ASVs with >0.5% abundance in at least 2 samples. Strong correlations are indicated with large squares, whereas weak correlations are indicated with small squares. The color of the scale bar denotes the nature of the correlation with 1 indicating a perfectly positive correlation (dark blue) and 1 indicating a perfectly negative correlation (dark red). Only significant correlations ($P < 0.05$) are shown.