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Exploitation of *Tenebrio molitor* larvae as biological factories for human probiotics, an exploratory study

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ABSTRACT

The exploitation of yellow mealworm (*Tenebrio molitor*) larvae for the bioaugmentation of probiotic *Bacillus clausii* strains was evaluated during a 7-day rearing period. qPCR was applied to evaluate the persistence and growth of *B. clausii* in the rearing substrate and larvae (washed and non-washed). Moreover, the effect of freeze-drying of larvae on *B. clausii* viability was evaluated. The results demonstrated the suitability of yellow mealworm as biological factories for the multiplication of *B. clausii* through a simple and inexpensive procedure, in view of the further application of larvae as foods and food ingredients. In more detail, an increase in the load of *B. clausii* was observed during the 7-day rearing of larvae fed wheat middlings spiked with 1 Log cells g^{-1}. Further research is needed to evaluate the most suitable technologies and processing parameters for obtaining yellow mealworm-based ingredients with a stable and active population of probiotic *B. clausii*.

1. Introduction

The exploitation of edible insects as an alternative protein source has recently attracted the attention of food business operators, research institutions and public bodies. The research on edible insects has concretely been boosted by the publication of the European Food Safety Authority (EFSA) Scientific Opinion on a risk profile related to the production and consumption of insects as food and feed (EFSA 2015) and by the issuing of Regulation (EU) 2015/2283 on novel foods. Indeed, following the EFSA Scientific Opinion, twelve insect species that can potentially be used as food and feed in the EU were identified. Moreover, Regulation (EU) 2015/2283 included edible insects in the so-called "novel foods" category and established a procedure for the commercialization of foods containing insects and their parts. In 2016, the EFSA Panel on Dietetic Products, Nutrition and Allergies (EFSA NDA Panel, 2016) issued guidance on the preparation and presentation of an application for the authorization of a novel food in the context of Regulation (EU) 2015/2283. The European Commission has recently issued the Implementing Regulation (EU) 2017/2469, laying down administrative and scientific requirements for applications referred to in Article 10 of Regulation (EU) 2015/2283.

In this context, if, on the one hand, the food industry explores the ability of edible insects to generate income, on the other hand, research institutions and public bodies have a mandate to evaluate the safety of this novel protein source (Mancini et al., 2019; Wynants et al., 2019). Amongst the above-mentioned actors, researchers also have the freedom and flexibility to investigate previously unexplored pathways to enhance the potential of edible insects as food or feed. It is noteworthy that, in the year 2021 the EFSA published a first Scientific Opinion on the safety assessment of dried yellow mealworm (*Tenebrio molitor* larva) as a novel food concluding that such food preparation is safe under the proposed uses and use levels. The EFSA Panel also noted that allergic reactions are likely to occur (EFSA NDA Panel, 2021). Among the edible insects features that have so far been evaluated, chemical and microbiological ones constitute the core of the available scientific literature (Garofalo et al., 2019; Rumpold & Schlüter, 2013; Schlüter et al., 2017). In more detail, regarding their microbiological evaluation, several studies have demonstrated that edible insects are natural carriers of various microorganisms. Indeed, as reviewed by Garofalo et al. (2019), the gut of edible insects can host mesophilic aerobes, bacterial endospores and spore-forming bacteria, Enterobacteriaceae, lactic acid bacteria, psychrotropic aerobes, and fungi. Moreover, the occurrence of potentially pathogenic and mycotoxigenic microbial species has been ascertained (Garofalo et al., 2019; Osimani & Aquilanti, 2021).

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Among the reported microorganisms, spore-forming bacteria represent a challenge for those who want to produce insect-based foods, since this bacterial group encompasses food-spoilage or potential pathogenic bacteria, including *Bacillus* spp. (Fasolato et al., 2018). So far, spores of *Bacillus* have frequently been found in insect-derived raw materials (e.g., insect powders) as well as ready-to-eat insect-based foods (Garofalo et al., 2019). Although the bacilli harbored by edible insects can represent a biological hazard and, hence, a risk for consumers (Fasolato et al., 2018), it is likely that even *Bacillus* strains with a beneficial health potential might survive and multiply in the gut of insects.

So far, strains of *Bacillus coagulans, Bacillus subtilis* and *Bacillus clausii* are being included in dietary supplements due to their ability of producing beneficial health effects in humans in accordance with the definition of probiotics (WHO/FAO 2006).

Regarding *B. clausii*, during sporulation, it releases antimicrobial substances that are active against Gram-positive microorganisms, including *Staphylococcus aureus, Enterococcus feacium*, and *Clostridium* ssp. (Urdaci, Bressollier, & Pinchuk, 2004). Moreover, strains of this species are naturally resistant to antibiotics, although this feature does not represent a safety issue if there is no risk of resistance transfer (Lakshmi, Jayanthi, Saravanan, & Ratna, 2017). *B. clausii* is also effective in alleviating the symptoms of diarrhea without causing any adverse effects (Patrone, Molinari, & Morelli, 2016). Probiotic strains of *B. clausii* are currently exploited in a number of commercially available formulations, including Enterogermina®, an "over-the-counter" (OTC) medicinal product registered in Italy in 1958 by Sanofi (Milan, Italy), recommended for the treatment of diarrhea, intestine-related disorders, respiratory tract issues, and bacterial imbalance due to antibiotic consumption (Coppi et al., 1985; Mazza, 1994).

Based on the above premises, spore-forming bacilli can reasonably be considered well-adapted to the gut environment of both humans and insects; this consideration prompted the proposal that edible insects could be successfully exploited as "living factories" for the multiplication of probiotic bacteria. In this regard, cell number augmentation and the maintenance of long-term viability are key aspects for the exploitation of cultures with a health benefiting potential (Bharti, Sharma, Saini, Verma, Nimonkar, & Prakash, 2017). Hence, the present study aimed to evaluate the exploitation of yellow mealworms (*Tenebrio molitor*) larvae for the bioaugmentation of probiotic *B. clausii* strains. To this end, a rearing substrate made of organic wheat middlings was spiked with a commercial *B. clausii* oral preparation (Enterogermina®) at two initial levels (low and high). Quantitative polymerase chain reaction (qPCR) analyses were performed to evaluate: i) the persistence and growth of *B. clausii* in the rearing substrate during the rearing period, as affected by the initial inoculation level; ii) the colonization and growth of the test microorganisms in the gut of the yellow mealworms; and iii) the effect of washing larvae on *B. clausii* loads. Moreover, the viability and eventual sporulation of *B. clausii* in freeze-dried larvae were evaluated by a culture dependent method followed by PCR detection of *B. clausii*.

2. Materials and methods

2.1. Experimental design

T. molitor larvae were reared on organic wheat middlings spiked with a low-level (1 Log cells g^{-1}) or a high-level (9 Log cells g^{-1}) load of probiotic strains of *B. clausii*. The suspension of spores used for the inocula was composed of four antibiotic resistant strains of *B. clausii,* each derived from ATCC 9799, a penicillin-resistant strain originally designated as *B. subtilis* (Cutting, 2011).

For each contamination level, two batches (each including 3 replicates) were set up, with one batch consisting of the wheat middlings spiked with *B. clausii* spores but no *T. molitor* larvae (control batch, CWM) and one consisting of the wheat middlings spiked with *B. clausii* spores and added *T. molitor* larvae (experimental rearing batch, RWM). Each replicate of the two batches was labeled with a unique alphanumeric code.

Larvae were sampled from each batch at regular intervals (1, 3 and 7 days) during the rearing period without any starvation prior sampling. Each sample was divided into two aliquots, one of which was washed with an ethanol aqueous solution whereas the other was analyzed without any washing. Regarding the washing treatment, larvae were given a surface disinfection treatment prior to microbial analysis in order to focus on the interior microbiota (Wynants et al., 2019)

Samples of the washed larvae collected at the end of the rearing period were subjected to freeze-drying, following a standard procedure.

Quantification of *B. clausii* cells and spores by qPCR was carried out on samples of: (i) the wheat middlings spiked with *B. clausii* spores collected from the CWM; (ii) fresh larvae (both washed and unwashed); and (iii) frass collected from the RWM.

The experimental design is depicted in Fig. 1. All details referring to each experimental phase are given in the sections below.

2.2. Subculturing of B. clausii and the inocula preparation

A spore suspension of four probiotic *B. clausii* strains (strain O/C, resistant to chloramphenicol; strain N/R, resistant to novobiocin and rifampicin; strain T resistant to tetracycline; and strain SIN resistant to neomycin and streptomycin) was supplied as Enterogermina® vials that were labeled as containing 8.6 Log spores mL^{-1} of *B. clausii*. So far, several beneficial health effects have been reported for the mixture of strains contained in this medicinal product (Abbrescia et al., 2014; Ripert et al., 2016). One mL of Enterogermina® oral suspension was inoculated twice in 20 mL Brain Heart Infusion (BHI) broth and incubated on a rotary shaker at 150 rpm for 24 h at 30 °C for spore germination and multiplication of the vegetative cells. The biomass was harvested by centrifugation (Rotofix 32 A, Hettich, Tuttlingen, Germany) at 4000 rpm for 5 min, after which the supernatant was discarded, and the cell pellet was resuspended in 20 mL sterile physiological solution (0.85% NaCl, w v^{-1}). The bacterial cells concentration was determined spectrophotometrically at 600 nm using a UV–Vis Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and the cell viability was checked by the spread plate method on BHI agar. To this end, two bacterial suspensions were prepared by diluting the initial suspension with sterile physiological solution (0.85% NaCl, w v^{-1}) to reach a final load of 1 and 9 Log cfu g^{-1}, respectively.

The simultaneous use of the 4 strains (O/C, SIN, N/R and T) of *B. clausii* included in Enterogermina® was in accordance with the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) recommendation regarding the need to use an inoculum composed of multiple strains (at least 2) when conducting microbiological food testing with artificially inoculated microorganisms (ANSES, 2019).

2.3. Feed

One batch of organic wheat middlings was purchased from a local mill factory (Molino Agostini s.r.L., Osimo, Italy) for use as a rearing substrate in the experimental trials. Organic carrots, used as a source of water for the larvae, were purchased from a local grocery store. Prior to the start of the rearing trial, the absence of *B. clausii* in both the wheat middlings and carrots was assessed as described in Section 2.7.

2.4. Bacillus clausii inoculation

The rearing trial was performed in aluminum trays (2.25 L, 5 × 21 × 27 cm) containing 240 g of the rearing substrate. To minimize any variation in the intrinsic properties of the rearing substrate that may have affected the growth characteristics of the inoculum (ANSES 2019), the latter component was set not to exceed 1% (v w^{-1}) of the test unit

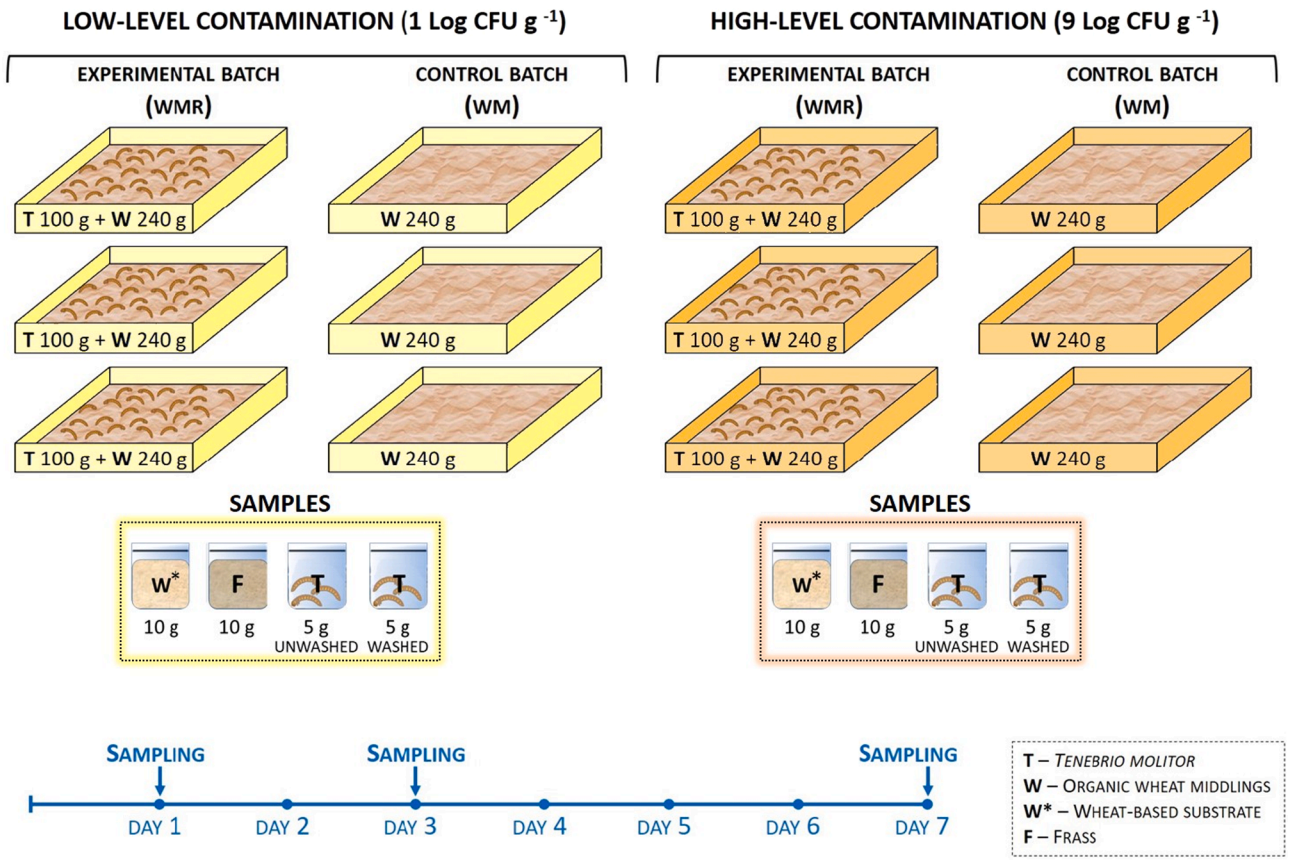

Fig. 1. Experimental design.

(240 g of rearing substrate). Aliquots (2.4 mL) of each *B. clausii* suspension (prepared as described in Section 2.2) were separately inoculated to reach an initial bacterial load of approximately 1 (low-level contamination) and 9 (high-level contamination) Log cfu g^{-1} of rearing substrate. The concentration of the resulting suspensions was checked by spread plate method on BHI agar plates. For each inoculum, 120 droplets (20 μL each) were uniformly distributed on the rearing substrate using a semiautomatic pipette. After inoculation, the rearing substrate was mixed for 5 min using a sterile spoon. To confirm the amount of each inoculum, 10 g aliquots of the rearing substrate were collected from each replicate of both the control and rearing batch immediately after inoculation (t0) and checked by qPCR method.

2.5. Yellow mealworm larvae rearing and sampling

Yellow mealworm larvae (penultimate and early stage of the last instar larvae) were purchased from a local pet shop (Moby Dick, Jesi, AN, Italy). All larvae were reared in accordance with the conditions already described by Belleggia et al. (2020). Briefly, prior to the start of the rearing trials, all larvae were fed with organic wheat middlings for 2 days in a climate-controlled chamber as described below. At the start of the trial, for each contamination level (each consisting of 3 replicates), 100 g of larvae were placed on the rearing substrate. Trays containing the control (CWM) and experimental (RWM) batches were placed in a climate-controlled chamber at 28 °C with a relative humidity of 60% for 7 days. Organic carrots that were previously washed and peeled under sterile conditions were supplied every 2 days to provide a water source.

Two aliquots (5 g each) of yellow mealworm larvae were collected at regular intervals (days 1, 3 and 7) during the rearing period from each batch. A sterilized standard No. 6 sieve (3.35 mm openings) was used to separate larvae from their frass. One aliquot of larvae was collected and directly subjected to further analysis (unwashed fresh larvae), whereas the remaining aliquot was first washed with a solution made with 70 mL 100% ethanol and 30 mL sterile deionized water and then subjected to further analysis (washed fresh larvae) (Belleggia et al., 2020). All of the collected larvae were frozen at − 20 °C for 40 min. In addition, at the same sampling times, aliquots (10 g) of frass and wheat middlings were also collected from each replicate of each batch, and all of the samples were placed into sterile bags for further analysis.

2.6. DNA extraction

An aliquot of each sample (10 g for wheat middlings and frass; 5 g for larvae) was tenfold diluted in a sterile physiological solution (0.85% NaCl, w v^{-1}) and homogenized for 5 min at 260 rpm using a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy). Ten milliliters of each sample homogenate (10^{-1} dilution) was centrifuged at 14000 rpm (Rotofix 32 A) for 10 min, the supernatants were discarded, and the cell pellets were used for the extraction of DNA using an E.Z.N.A. Soil DNA Kit (Omega Biotek, Norcross, GA, U.S.A.) following the manufacturer's instructions. Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, U.S.A.) was used to determine the quantity and the purity of the extracted DNAs, which were then standardized to a concentration of 15 ng μL^{-1} before further analysis.

2.7. qPCR quantification of B. clausii

Absolute quantification of *B. clausii* in the wheat middlings, *T. molitor* larvae, and frass was performed by qPCR in a Mastercycler® ep realplex machine (Eppendorf, Hamburg, Germany). The forward (5′-AATTTTACCGCCCCTCAAG-3′) and reverse (5′-ACTTTTGGAACATGCCGAAC-3′) primers used for the amplification of the bacterial *erm34* gene conferring resistance to macrolides and highly specific for *B. clausii* were previously designed by Perotti et al. (2006). Standard curves were

created using both spores and vegetative cells. Ten-fold serial dilutions of *B. clausii* spores were prepared starting from the Enterogermina® vial containing 8.6 Log spores mL^{-1} of *B. clausii*. The suspension of vegetative cells was prepared according to the procedure described above and further 10-fold serially diluted. The enumeration of vegetative cells in the nondiluted suspension was performed by the plate count method on BHI agar. DNA was extracted from 1 mL of each dilution (spores and vegetative cells) using the E.Z.N.A. Soil DNA Kit as previously described. To further analyze the sensitivity of the method under the applied experimental conditions and the efficiency of the kit used for the extraction of the DNA, aliquots (2 g) of sterilized wheat middlings were spiked with 1 mL of each 10-fold serial dilution of both spores and vegetative cells and mixed manually for 5 min. DNA was extracted from 250 mg of each inoculated wheat middlings sample. The quantity and the purity of the extracted DNAs were checked by Nanodrop ND 1000 (Thermo Fisher Scientific). To create standard curves, each qPCR reaction mixture (total volume 10 μL) consisted of 4 μL of the extracted DNA; 5 μL of Type-it 2X HRM PCR Master Mix (containing HotStarTaq Plus DNA Polymerase, EvaGreen Dye, an optimized concentration of Qsolution, dNTPs and $MgCl_2$) (Qiagen, Hilden, Germany); and 500 nM of each primer. The thermal cycling program was as follows: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 1 min at 62 °C, and 72 °C for 20 s. The specificity of the amplification reaction was checked by melting curve analysis performed with the temperature gradually increasing from 65 °C to 95 °C by 0.4 °C s^{-1}. For some randomly selected amplicons, the specificity was also confirmed by sequencing and alignment to those deposited at the GenBank database (https://pubmed.ncbi.nlm.nih.gov/).

The standard curves were constructed by plotting the Ct values of the qPCR performed on the extracted DNAs against the number of spores or vegetative cells per reaction. The qPCR amplification efficiencies (E) and the correlation coefficients (R^2) were calculated automatically by Mastercycler® ep realplex software from the slope of the standard curves (Stolovitzky & Cecchi, 1996). All standard curves were created covering the range from 1 to 6 Log cells or spores of *B. clausii* per reaction in order to determine the qPCR detection limit.

For the absolute quantification of *B. clausii* vegetative cells or spores in the larvae (washed and non-washed), wheat middlings and frass, the extracted DNAs were run together with the 10-fold serial dilutions of the standards prepared as described above. The load of *B. clausii* in the analyzed samples was determined using the slope of the standard curves. All qPCR reactions were performed in triplicate and the results were reported as the mean value (expressed as Log cells/spores per g of sample) ± standard deviation.

2.8. Tenebrio molitor larvae freeze-drying

To assess the survival of *B. clausii*, 15 g of washed *T. molitor* larvae collected at the end of the rearing period (t7) were subjected to freeze-drying using a VirTis freeze dryer (SP Scientific, Warminster, PA, USA). Freeze-drying of larvae was chosen to evaluate the survival of *B. clausii* during a possible long-term storage of insects prior their further processing (heat treatment). The treated larvae were subjected to viable cells and spore counting as described in Section 2.9.

2.9. Determination of Bacillus clausii viability in Tenebrio molitor larvae

The viability and eventual sporulation of *B. clausii* in the fresh *T. molitor* larvae at the end of the experimental trial (t7), as well as in the freeze-dried larvae, were checked by a culture dependent method followed by PCR detection of *B. clausii*. Four pools of the samples were prepared, namely: i) pools M1 and M9, consisting of the fresh larvae initially spiked with 1 and 9 Log cell g^{-1}, respectively; and ii) pools ML1 and ML9, consisting of the freeze-dried larvae initially spiked with 1 and 9 Log cell g^{-1}, respectively. All pools were prepared by weighing 3.33 g of the sample from each experimental replicate for a total weight of 10 g, which was then mixed with 90 mL of sterile physiological solution (0.85% NaCl, w v^{-1}) and homogenized as described above. For the detection of the bacterial spores, an aliquot (1 mL) of each homogenate was subjected to heat treatment (80 °C for 15 min) in a temperature-controlled water bath for the removal of the vegetative cells (Milanović et al., 2017). The homogenates were serially diluted, inoculated on BHI agar plates and incubated at 30 °C for 48 h. Bulk cells were collected from each plate containing at least 1 colony forming unit as previously described by Osimani et al. (2018a). DNA was extracted from the bulk cells using the E.Z.N.A. Soil DNA Kit as described above and amplified via PCR using the primers and conditions described in Section 2.7. The results are expressed as the presence (+) or absence (-) of *B. clausii* (vegetative cells and spores) in each dilution BHI plate.

2.10. Water activity measurement

The water activity of the wheat middlings and frass (a_w) was measured in accordance with the ISO 21807:2004 standard method using an Aqualab 4 TE apparatus (Meter Group, Pullman, USA).

2.11. Statistical analysis

A completely randomized design, with three replicates, was applied using time, inoculum level, and feeding substrate (wheat middlings) as independent factors; data were analyzed using a fixed model three-way ANOVA with two and three-way interactions. The Tukey's HSD (Honestly Significant Differences) multiple comparisons procedure was performed to test differences among means. Time 0 (t0) was not considered since it corresponded in all samples to the day of *B. clausii* inoculation. All statistical analyses were performed using JMP 11.0 software (SAS Inc.).

3. Results and discussion

To date, spore forming bacteria have been acknowledged as one of the major safety issues regarding the consumption of edible insects (Garofalo et al., 2019). Although this assumption remains valid, the present study explored, for the very first time, the exploitability of yellow mealworms as biological factories for the multiplication of probiotic strains of *B. clausii*, in view of the further application of larvae as either feed additives or foods and food ingredients.

Species of *Bacillus* (e.g., *Bacillus thuringiensis*) are known to exert biological insecticidal effects (Eski, Demir, Güllü, & Demirbağ, 2018). However, in the present study, no mortality was observed in *T. molitor* larvae treated with probiotic strains of *B. clausii* throughout the 7-day rearing period, thus suggesting that the occurrence of *B. clausii* in the rearing substrate did not affect the viability of yellow mealworms, irrespective of the initial contamination level.

Additionally, a stable viability of *B. clausii* in the rearing substrate was seen throughout the monitoring period. In 2017, Zhong et al. first demonstrated the suitability of a simple and inexpensive procedure for the massive growth of a mixed culture of *Bifidobacterium bifidum*, *Clostridium butyricum*, *Bacillus subtilis* and *Bacillus licheniformis* on a solid-state medium generated by pouring a liquid MRS medium onto wheat bran. The findings collected in the present study agree with those of Zhong et al. (2017), who clearly demonstrated the exploitability of a probiotic feed additive for feeding of yellow mealworms.

In the present study, a qPCR protocol was used for the absolute quantification of *B. clausii* in the wheat middlings, *T. molitor* larvae and frass. The standard curves, generated by plotting the Ct values of the qPCR performed on 10-fold serial dilutions, showed R^2 values of 1.000 and 0.998 and efficiencies of 1.09 and 1.01 for *B. clausii* spores and vegetative cells, respectively. The specificity of the amplification reaction checked by melting curve analysis showed that all PCR products had a melting temperature comprised between 85.2 and 85.6 °C. The detection limit, defined as the lowest number of detectable *B. clausii*

vegetative cells or spores per reaction, was 2 Log cells or spores. This latter finding is consistent with the results of a previous study using the same primer pair for the quantification of *B. clausii* cells in biological samples (Perotti et al., 2006). The single-copy chromosomal gene *erm34*, encoding resistance to macrolides, was selected as a target for the amplification reactions due to its acknowledged specificity for *B. clausii* (Bozdogan, Galopin, & Leclercq, 2004).

Moreover, to check the robustness of the quantification assay and the efficiency of the DNA extraction kit (E.Z.N.A. Soil DNA Kit), aliquots of sterilized wheat middlings were separately inoculated with 10-fold serial dilutions of *B. clausii* spores and vegetative cells. The resulting qPCR standard curves showed a very good R^2 (0.998 and 0.997) and efficiency (1.05 and 0.90) for both the spores and vegetative cells, respectively. The successful DNA extraction from *B. clausii* using the E.Z.N.A. Soil DNA Kit confirms what was previously reported by Dineen, Aranda, Anders, and Robertson (2010), who used the same commercial kit for DNA extraction from bacterial spores followed by qPCR, thus once more emphasizing the impact of nucleic acids extraction systems on the success of qPCR analyses.

The results of the descriptive statistical analysis of qPCR quantification of *B. clausii* in the feeding substrate, *T. molitor* larvae and frass are reported in Table 1.

Regarding the control wheat middlings initially inoculated with 1 Log cell g^{-1}, *B. clausii* was never detected by qPCR during the monitoring period, suggesting that the substrate alone was not able to sustain the growth of the tested microorganisms. Indeed, the a_w values of the wheat middlings ($\leq$0.62) in both the RWM and CWM batches as well as the frass were not compatible with the growth of *Bacillus* spp., which requires a minimum a_w of 0.92 (Osimani et al. 2018b). Moreover, at t7 in the wheat middlings used for rearing, an average *B. clausii* load of 2.84 $\pm$ 0.16 Log cells g^{-1} was detected (Table 1). The occurrence of *B. clausii* below the detection limit in the samples collected at t0 and t3 did not allowed ANOVA to be performed.

As for the wheat middlings with an initial high-level contamination, mean values between 9.38 $\pm$ 0.44 and 9.51 $\pm$ 0.15 Log cells g^{-1} were detected during the monitoring period in both of the analyzed batches, thus confirming the proper inoculation of the wheat middlings to the desired loads of the test microorganisms.

Given the qPCR results, the ANOVA was exclusively performed using the dataset referred to the wheat middlings initially spiked with 9 Log cells g^{-1} of *B. clausii*. The analysis of these data showed that the two main factors Time and Feeding substrate were significant, as well as the two-way interactions (Table 2).

As shown in Fig. 2 (panel a), a trend showing a slight decrease of *B. clausii* cells and spores was observed from t1 to t7, with a significantly lower mean value (8.80 Log cells g^{-1}) at t7 than t1 (9.20 Log cells g^{-1}). Unexpectedly, a significantly lower mean load of *B. clausii* was observed in the RWM than that in the CWM (Fig. 2, panel b).

The Time by Feeding substrate interaction (Fig. 2, panel c) showed that for the CWM, the counts of *B. clausii* remained almost unchanged overtime, whereas for RWM, they progressively decreased from t1 to t7. In more detail, for the *B. clausii* cells detected in the RWM, the mean load at t1 (9.01 Log cells g^{-1}) was significantly higher than that at t7 (8.09 Log cells g^{-1}) with an intermediate mean value reached at t3. Furthermore, at t1, the loads found for the RWM and CWM were not significantly different, 8.65 and 9.49 Log cells g^{-1}, respectively; moreover, at t3 and t7, significant differences were observed between the RWM and CWM, with mean counts at 8.09 and 9.50 Log cells g^{-}1, respectively.

Regarding *T. molitor*, at t1 no *B. clausii* cells were detected by qPCR in the larvae initially spiked with 1 Log cell g^{-1} (Table 1); for this reason,

Table 2
ANOVA results for *Bacillus clausii* quantified by qPCR in samples of control inoculated feeding and mealworm larvae rearing substrates.

Source of variation	df	Means Square	
Time (T)	2	1.485	*
Feeding Substrate (F)	1	10.340	***
T × F	2	2.466	**
Error	12	1.547	***
Sampling error	36	0.260	

Time = 3 levels (day 1; day 3; day 7).
Feeding Substrate = 2 levels (1 = Control Inoculated Wheat middlings; 2 = Inoculated Wheat middlings for rearing).
* significant $P < 0.05$; ** significant $P < 0.001$; *** significant $P < 0.0001$.

Table 1
Results of qPCR quantification of *Bacillus clausii* (Log cells g^{-1}) in organic wheat middlings from the control batch (CWM), frass (organic wheat middlings mixed with insects' excrements) from the experimental batch (RWM), unwashed mealworm larvae (UL) and washed mealworm larvae (L).

IL	Sample	Replicate 1				Replicate 2				Replicate 3				Overall means			
		t0	t1	t3	t7	t0	t1	t3	t7	t0	t1	t3	t7	t0	t1	t3	t7
1	CWM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d	n.d	n.d
	RWM	n.d.	n.d.	n.d.	2.93 ± 0.13	n.d.	n.d.	n.d.	2.66 ± 0.35	n.d.	n.d.	n.d.	2.94 ± 0.12	n.d	n.d	n.d	2.84 ± 0.16
	UL	n.d.	n.d.	2.90 ± 0.07	3.13 ± 0.07	n.d.	n.d.	2.85 ± 0.03	3.27 ± 0.15	n.d.	n.d.	2.93 ± 0.03	3.35 ± 0.14	n.d	n.d	2.89 ± 0.04	3.25 ± 0.11
	L	n.d.	n.d.	2.87 ± 0.03	3.25 ± 0.16	n.d.	n.d.	2.89 ± 0.09	3.14 ± 0.05	n.d.	n.d.	2.82 ± 0.04	3.13 ± 0.30	n.d	n.d	2.86 ± 0.04	3.17 ± 0.07
9	CWM	9.45 ± 0.00	9.67 ± 0.07	9.54 ± 0.04	9.39 ± 0.10	9.71 ± 0.04	9.59 ± 0.07	9.37 ± 0.01	9.45 ± 0.08	9.46 ± 0.07	8.87 ± 0.12	9.56 ± 0.05	9.68 ± 0.18	9.54 ± 0.15	9.38 ± 0.44	9.49 ± 0.11	9.51 ± 0.15
	RWM	9.76 ± 0.06	8.98 ± 0.04	8.52 ± 0.10	8.19 ± 0.09	9.74 ± 0.03	9.07 ± 0.04	8.79 ± 0.02	8.05 ± 0.04	9.82 ± 0.08	8.99 ± 0.08	8.64 ± 0.03	8.03 ± 0.15	9.77 ± 0.04	9.01 ± 0.05	8.65 ± 0.13	8.09 ± 0.09
	UL	n.d.	6.95 ± 0.04	5.58 ± 0.17	6.51 ± 0.09	n.d.	6.87 ± 0.02	5.85 ± 0.09	6.05 ± 0.06	n.d.	7.20 ± 0.09	5.54 ± 0.17	6.30 ± 0.02	n.d	7.00 ± 0.17	5.66 ± 0.17	6.29 ± 0.23
	L	n.d.	6.91 ± 0.01	5.29 ± 0.39	5.80 ± 0.25	n.d.	6.33 ± 0.10	5.35 ± 0.08	6.11 ± 0.14	n.d.	6.22 ± 0.10	5.41 ± 0.16	5.93 ± 0.23	n.d	6.49 ± 0.37	5.35 ± 0.06	5.95 ± 0.15

IL, inoculum level (Log cfu g^{-1}).
Values are expressed as mean log cells g^{-1} $\pm$ standard deviation.
t0, day of inoculation; t1, day 1; t3, day 3; t7, day 7. n.d., not detected.

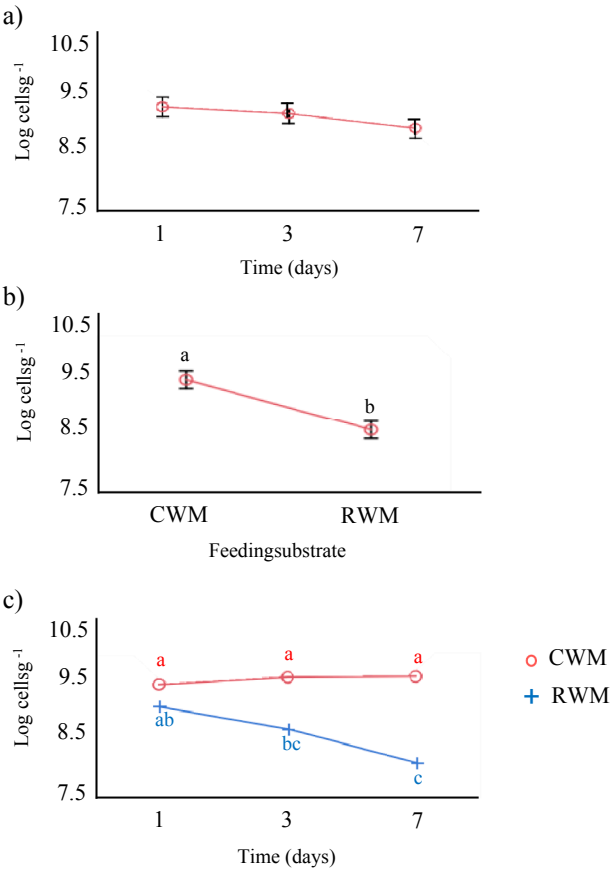

Fig. 2. Load (expressed as mean values of Log cells g^{-1} calculated on the basis of all the replicates of both the inoculation levels) of *Bacillus clausii* assessed by qPCR in control wheat middling (CWM) and inoculated wheat middlings for mealworm larvae rearing (RWM) at 9 Log cells g^{-1} inoculum level, analysed according to the two main factors, Time (T) (panel a), Feeding substrate (F) (panel b), and first order interaction, namely T × F (panel c). Within each panel, different letters indicate significant differences according to the Tukey's HSD test ($P < 0.05$).

the ANOVA was exclusively carried out on qPCR data collected at t3 and t7.

The statistical analysis showed that all of the main factors, being Time, Inoculum and Washing treatment, were significant, as well as the two-way interactions Time × Inoculum and Inoculum × Washing treatment (Table 3).

Table 3
ANOVA results for *Bacillus clausii* quantified by qPCR in samples of unwashed and washed mealworm larvae.

Source of variation	df	Means Square	
Time (T)	1	4.028	***
Inoculum (I)	1	138.085	***
Washing treatment (W)[#]	1	0.652	**
T × W	1	0.005	n.s.
T × I	1	0.343	*
I × W	1	0.319	*
T × I × W	1	0.000	n.s.
Error	16	0.764	**
Sampling error	48	0.260	

Time = 2 levels (day 3; day 7).
Inoculum = 2 levels (1 = 1 log cfu g^{-1}; 9 = 9 log cfu g^{-1}).
Washing treatment = 2 levels (1 = Unwashed mealworm larvae; 2 = Washed mealworm larvae).
* significant $P < 0.05$; ** significant $P < 0.001$; *** significant $P < 0.0001$; n.s. not significant.
[#] larvae washed with ethanol solution in sterile deionized water.

As shown in Fig. 3 (panel a), a slight but significantly increasing trend was observed for the load of *B. clausii* from t3 to t7, at 4.19 and 4.86 Log cells g^{-1}, respectively.

As for the Inoculum main factor, as expected, *B. clausii* counts significantly increased in larvae with an initial high-level contamination, showing a mean value of 5.81 Log cells g^{-1}, in respect to larvae with an initial low-level contamination (Fig. 3, panel b).

Regarding the washing treatment effect, a minimal but significant reduction in *B. clausii* cell count (−0.19 Log cells g^{-1}) was observed in the larvae subjected to washing treatment with the ethanol solution in sterile deionized water (Fig. 3, panel c). The low reduction in *B. clausii* cell counts after washing can be explained by the different distribution of the microorganism in the larvae, being *B. clausii* likely more present in the intestine rather than on the cuticle.

As shown in Fig. 3, panel d, a significantly higher increase in the load of *B. clausii* was shown by the larvae with an initial high-level inoculum (from 5.51 to 6.18 Log cells g^{-1} at t3 and t7, respectively) than those with a low-level inoculum (from 2.87 to 3.21 Log cells g^{-1} at t3 and t7, respectively). Moreover, at both t3 and t7, the load of *B. clausii* was significantly higher in the larvae spiked with 9 Log cells g^{-1}.

Concerning the Inoculum × Washing treatment interaction (Fig. 3, panel e), the larvae with an initial high-level inoculum showed significantly higher *B. clausii* counts than those spiked with a low level of the test microorganisms, irrespective of the washing treatment. The significance of the first order interaction between Inoculum and Washing treatment (Fig. 3, panel e) reflected a different effect of the washing treatment at the two inoculation levels. In more detail, for the larvae spiked with 1 Log cells g^{-1}, the washing treatment did not significantly affect the load of *B. clausii*, whereas a significant decrease in the load of the test microorganisms was observed after washing of the larvae initially spiked with 9 Log cells g^{-1}. Notwithstanding, the results obtained in the present study suggest that *B. clausii* cells and spores are harbored in the larval gut rather than the external cuticle.

Since no selective growth media are available for the viable counting of *B. clausii*, the BHI growth medium was used for the enumeration of the test microorganisms and bulk colonies preparation.

The results of the assessment of *B. clausii* viability and spore counts in *T. molitor* larvae at t7 are reported in Table 4. In more detail, for the larvae reared on the wheat middlings spiked with 1 Log cells g^{-1}, viable cells up to 3 Log cells g^{-1} were detected, whereas spore counts were < 1 Log cells g^{-1}. For the freeze-dried larvae, the counts of viable cells were at approximately 2 Log cells g^{-1}, whereas those of the spores were, as expected, < 1 Log cells g^{-1}.

As for the larvae reared on the wheat middlings initially spiked with 9 Log cells g^{-1}, after 7-day rearing, the viable cells were 6 Log cells g^{-1}, whereas spore counts were approximately 2 Log cells g^{-1}. Finally, for the freeze-dried larvae, the counts of the viable cells were 5 Log cells g^{-1}, whereas those of the spores were 1 Log cells g^{-1}.

As the results of qPCR quantification of *B. clausii* cells are considered, a progressive decrease was seen in the larvae fed wheat middlings with the high-inoculation level. This result might tentatively be ascribed to a limiting effect exerted by the insect gut resident microbiota. This hypothesis is supported by what reported by Chellaram et al. (2012) about the production by insect gut associated bacteria of secondary metabolites with broad-spectrum antimicrobial properties. At this regard, further research is needed to shed light on the interactions of *B. clausii* with yellow mealworms gut microbiota as well as on the potential establishment of positive microbial associations favoring the propagation and yield of *B. clausii*.

As far as the results collected on freeze-dried larvae are concerned, as reported by Han et al. (2018), freeze-drying can damage microbial cells due to ice crystal formation and cell membrane injury, thus likely explaining the observed reduction in viability of *B. clausii* in the larvae subjected to this treatment resulted after culture dependent method followed by PCR detection of *B.clausii*.

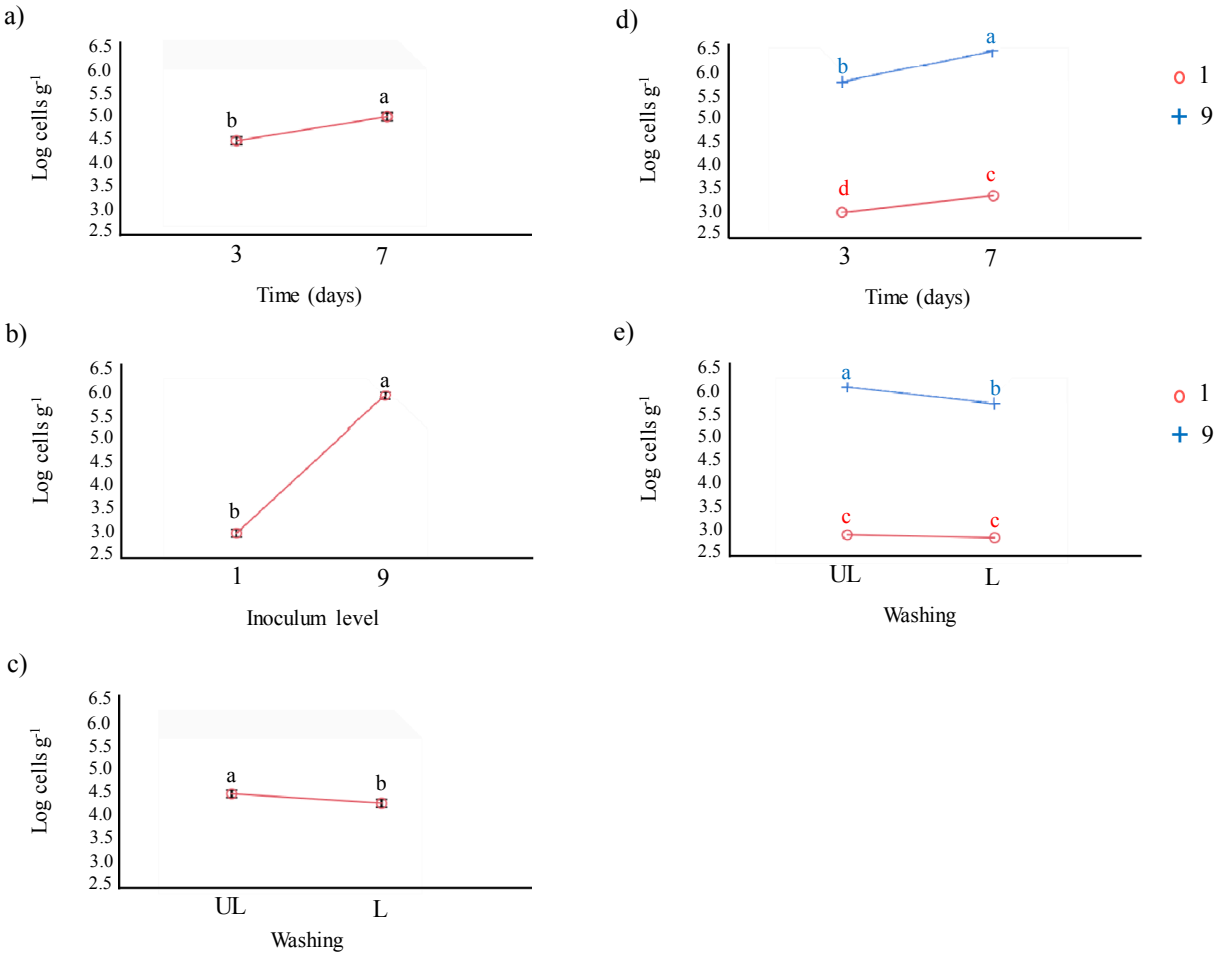

Fig. 3. Load (expressed as mean values of Log cells g^{-1} calculated considering all the replicates of both the inoculation levels) of *Bacillus clausii* assessed by qPCR in unwashed (UL) and washed mealworm larvae (L), analysed according to the three main factors, Time (T) (panel a), Inoculum (I) (panel b), Washing treatment (W) (panel c), first order interactions, namely T × I (panel d) and I × W (panel e). Within each panel, different letters indicate significant differences according to the Tukey's HSD test ($P < 0.05$). 1, inoculum level 1 Log cells g^{-1}; 9, inoculum level 9 Log cells g^{-1}.

Table 4
Determination of *Bacillus clausii* viability and eventual sporulation in *Tenebrio molitor* larvae at the end of the experimental trial (t7).

Samples		BHI agar plates (dilutions)						
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
M1	vegetative cells	+	+	+	-	n.a	n.a	n.a
	spores	–	–	–	–	n.a	n.a	n.a
ML1	vegetative cells	+	+	–	–	n.a	n.a	n.a
	spores	–	–	–	–	n.a	n.a	n.a
M9	vegetative cells	+	+	+	+	+	+	–
	spores	+	+	–	–	–	–	–
ML9	vegetative cells	+	+	+	+	+	–	–
	spores	+	–	–	–	–	–	–

BHI, Brain Heart Infusion; M1, pool of *T. molitor* larvae with low-level *B. clausii* inoculum (1 Log cells g^{-1}); ML1, pool of freeze-dried *T. molitor* larvae with low-level *B. clausii* inoculum (1 Log cells g^{-1}); M9, pool of *T. molitor* larvae with high-level *B. clausii* inoculum (9 Log cells g^{-1}); ML9, pool of freeze-dried *T. molitor* larvae with high-level *B. clausii* inoculum (9 Log cells g^{-1}); +, *B.clausii* detected by PCR; -, *B.clausii* not detected by PCR; n.a., not applied.

The addition of probiotic cultures to foods is not a novelty *per se*; however, most probiotic foods currently available on market consist of dairy products harboring probiotic strains of lactic acid bacteria or bifidobacteria (Sarkar & Mandal, 2016; Shori, 2017). In this context, functional foods enriched with probiotic *Bacillus* spp. are definitely less popular, notwithstanding the acknowledged features of these spore formers, such as their high acid tolerance, good stability during heat processing, dehydration and low temperature storage, and survivability under hostile environments (Elshaghabee, Rokana, Gulhane, Sharma, & Panwar, 2017; Lakshmi et al., 2017; Mazza, 1994). Additional beneficial properties of probiotic bacilli include the production of antioxidant compounds, vitamins, antimicrobial peptides, and small extracellular effector molecules (Elshaghabee et al., 2017; Lee, Kim, & Paik, 2019). Given these findings, the exploitation of human probiotic spore formers for the feeding of edible insects to be used as feed or food undoubtedly constitutes an absolute novelty.

Very recently, *T. molitor* larvae have successfully been exploited as carriers of probiotic lactic acid bacteria for the production of poultry

feed (Islam & Yang, 2017). The encouraging results achieved by these authors seem to support the vision that has inspired the present study, aiming at exploiting yellow mealworms as potential carriers of probiotic bacilli, with feed or food applications. Regarding these latter, to date numerous insect-derived ingredients and insect-containing foods have been developed by food-industry operators and researchers. Bread (González, Garzón, & Rosell, 2019; Osimani et al., 2018c; Roncolini, Milanović, Cardinali, Osimani, Garofalo, Sabbatini, Clementi, Pasquini, Mozzon, Foligni, Raffaelli, Zamporlini, Minazzato, Trombetta, Van Buitenen, Van Campenhout, & Aquilanti, 2019; Roncolini, Milanović, Aquilanti, Cardinali, Garofalo, Sabbatini, Clementi, Belleggia, Pasquini, Mozzon,Foligni, Trombetta, Haouet, Altissimi, Di Bella, Piersanti, Griffoni, Reale, Niro, & Osimani, 2020), biscuits (Homann, Ayieko, Konyole, & Roos, 2017), pasta (Duda, Adamczak, Chełmińska, Juszkiewicz, & Kowalczewski, 2019), muffins (Pauter et al., 2018) and cookies (Terry, Lupul, & Coate, 2017) are among the promising insect-containing foods that could take advantage of the exploitation of insect-derived ingredients carrying heat-resistant spores of probiotic bacilli.

Of note, the results previously reported by Wynants et al. (2017), who suggested that starvation and rinsing of mealworms at the end of the rearing process have no significant effects on both the load and composition of the bacterial community of treated (starved, rinsed or subjected to a combination of both treatments) and untreated larvae. This evidence supports the assumption that the practices commonly applied by the insect-rearing industry to enhance the microbial quality of insect larvae for human consumption are not expected to significantly affect the load and viability of probiotic *B. clausii* spores carried in the insect gut.

4. Conclusions

The results overall collected in the present study demonstrated the suitability of *T. molitor* larvae as biological factories for the multiplication of probiotic strains of *B. clausii*. An appreciable increase in the load of the tested microorganisms was observed during the 7-day rearing of larvae fed wheat middlings spiked with 1 Log cells g^{-1}, thus suggesting that a further increase of the bacterial load might potentially be achieved with a prolonged rearing time. However, it should be also noted that as a high-level inoculation (9 Log cells g^{-1}) was assayed, a progressive decrease in the load of *B. clausii* was seen during the 7-day rearing, with final cell numbers attesting at around 6 Log g^{-1} in fresh larvae.

Moreover, differences between washed and unwashed larvae were seen, depending on the inoculum level.

Finally, the results collected onto freeze-dried larvae suggested that, under the conditions applied, freeze-drying does not represent a valid choice for processing of yellow mealworms enriched with *B. clausii* spores. Indeed, to exert its probiotic features, *B. clausii* need to survive transit through the human gastrointestinal tract in the form of a spore and there to undergo germination, outgrowth, and further multiplication as vegetative forms.

Open questions remain regarding the efficiency of propagation of *B. clausii* in the gut of yellow mealworms as well as the most suitable technologies and processing parameters for obtaining *T. molitor*-derived food ingredients (e.g., powdered larvae) and/or *T. molitor*-containing foods with a stable and active population of probiotic *B. clausii.*

The very promising results achieved by Zhong et al. (2017) on *T. molitor* larvae fed wheat bran enriched with a mixture of probiotic cultures, including *B. subtilis* and *B. licheniformis,* encourage further research efforts also for the assessment of the effect of *B. clausii* on growth performance and feed conversion efficiency of mealworms.

It is noteworthy that, in insects, nutrient uptake and digestion are regulated by the gut-brain axis which is a neurohumoral communication system for maintaining gut homeostasis. In the insect gut, the interaction of signaling molecules (e.g., hormones) regulates a multitude of processes including gut physiology (Abou El Asrar, Cools, & Vanden Broeck, 2020). Moreover, it is known that insect gut microbiota plays a key role in host nutrition, detoxification of toxic compounds, or reproduction (Muñoz-Benavent, Pérez-Cobas, García-Ferris, Moya, & Latorre, 2021), hence, further research is needed to evaluate the effect of the inoculated *B. clausii* on the insect gut physiology and the interactions occurring among the test microorganism and the insect gut microbiota.

Ethic statement

This study employed *Tenebrio molitor* larvae as model organism and has not involved any human subjects.

CRediT authorship contribution statement

Vesna Milanović: Investigation, Formal analysis. **Federica Cardinali:** Formal analysis. **Luca Belleggia:** Investigation. **Cristiana Garofalo:** Investigation, Formal analysis. **Marina Pasquini:** Formal analysis. **Stefano Tavoletti:** Formal analysis. **Paola Riolo:** Investigation, Formal analysis. **Sara Ruschioni:** Investigation, Formal analysis. **Nunzio Isidoro:** Resources. **Andrea Osimani:** Conceptualization, Writing - review & editing, Supervision. **Lucia Aquilanti:** Conceptualization, Writing - review & editing.

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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