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Listeria monocytogenes contamination of *Tenebrio molitor* larvae rearing substrate: Preliminary evaluations

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Abstract

Today, edible insects represent a hot topic as an emerging and eco-friendly source of protein. The mealworm (*Tenebrio molitor* L.) is among the most employed insects for human consumption and feed purposes. So far *Listeria monocytogenes*, have never been detected either in products sold on the market or during the rearing process. In this study, the substrate employed for mealworm rearing was deliberately contaminated with *L. monocytogenes* and the bacterium was enumerated during the rearing period and after technological treatments of the larvae. *L. monocytogenes* persisted during the rearing period. Washing the larvae did not produce any significant effect, while fasting the larvae for 24 or 48 hours reduced the *L. monocytogenes* load ($P < 0.001$). Oven cooking eliminated *L. monocytogenes* cells from the product, reducing the risk associated to this foodborne pathogen to zero.

Keywords: mealworm; fasting; washing; cooking; pupae; edible insects.

1. Introduction

Today, insects are one of the hot topics in animal science and food science for their potential employment as feed and for human consumption. Even though several cultures accept edible insect as part of their diet, this practice is relatively recent in western countries (Hartmann et al., 2015; Mancini et al., 2019b; Tan et al., 2016). If, on one hand, edible insects could be a practical answer to the increasing request for animal proteins with an environmental impact lower than that related to conventional livestock (Oonincx and de Boer, 2012; van Huis et al., 2013) and with nutritional values comparable (Rumpold and Schlüter, 2014) to those of traditional reared animals, on the other hand, further research is needed to offer the market a product free from hazards (Belluco et al., 2013).

Research articles on the microbiological evaluation of edible insects have reported a great variability in microbial loads, with differences mainly due to insect type or origin and the technological processing of the products (Garofalo et al., 2017; Grabowski and Klein, 2017b, 2017a; Klunder et al., 2012; Mancini et al., 2019a; Osimani et al., 2018; Stoops et al., 2017, 2016; Vandeweyer et al., 2017a, 2017b; Wynants et al., 2018, 2017).

Notably, encouraging results have been reported concerning the determination of pathogenic bacteria in edible insects. More specifically, *Salmonella* spp. and *Listeria* spp. and *Bacillus cereus* were never detected during rearing period or in insects intended for processing; only a few researchers detected coagulase positive staphylococci in insects reared for feed/food purposes in a controlled environment (lab scale farming or food industries). The contamination of the rearing substrate is certainly one of the main factors that might affect the microbial contamination. Banjo et al. (2005) and Banjo et al. (2006) detected *B. cereus* and *Staphylococcus aureus* in domestic housefly larvae (*Musca domestica*) cultured on fresh fish and in African rhinoceros beetles (*Oryctes monocerus*) collected in Nigeria.

Furthermore, beside rearing practices, microbial load could derive from slaughtering procedures and processing (EFSA, 2015). Several research studies reported detection of foodborne pathogens, such as presumptive *Bacillus cereus* and *Listeria* spp., in ready-to-eat products sold on the market (Fasolato et al., 2018; NVWA, 2014; Osimani et al., 2017).

Listeria monocytogenes is a saprophyte microorganism and, due to its ubiquitous nature, it could possibly occur in the rearing environment and consequently heavily compromise the product safety.

L. monocytogenes is indeed able to survive for a long time in the environment and contaminate water, soil, silage, vegetables, fruits and several foods of animal origin, such as dairy products, raw or cooked meat and seafood (O'Connor et al., 2010). It can be responsible for a serious form of disease, listeriosis, which is currently considered one of the major foodborne illnesses in the world (Välimaa et al., 2015). In fact, while the disease manifests itself in most cases as a mild febrile form, a systemic form featuring more severe nervous symptomatology causing high rates of hospitalization and even death can also occur(Buchanan et al., 2017).

To the best of our knowledge, no data are available on the factual possibility that reared insects might be contaminated by *L. monocytogenes* during the farming process.

For these reasons, the aim of this study was to evaluate the persistence of *L. monocytogenes* in *Tenebrio molitor* L. larvae reared on a deliberately contaminated substrate. Procedures such as fasting, washing and cooking were also evaluated as technological treatments to reduce *L. monocytogenes* loads.

2. Material and methods

2.1. Experimental design

Mealworms (*T. molitor* L. 1758; Coleoptera Tenebrionidae) were reared in plastic containers (39 × 28 × 14 cm) at the Department of Veterinary Sciences (University of Pisa, Italy) under a laboratory scale production (temperature: 25 °C; relative humidity: 55-65 %). A mix 1:1 of brewer's spent grain and bread was used as substrate (dry matter, DM: 96%; ether extract: 2.27% on DM; crude protein: 14.56% on DM; ash: 2.45% on DM).

Two different experimental trials were designed in order to (i) evaluate *L. monocytogenes* persistence/enumeration and (ii) test the effectiveness of technological treatments (fasting for 24 or 48 h, washing and oven cooking).

Preliminary analyses were performed in order to verify the absence of the microorganism in the rearing substrate and in the larvae.

2.2. Bacterial strain and *Listeria monocytogenes* enumeration

L. monocytogenes ATCC 7644 was employed in all trials. The strain was stored at −80 °C in a glycerol suspension until its use and cultured in BHI (Brain and Hearth Infusion, Thermo Fisher Scientific, Milan, Italy) broth for 24 h at 37 °C in aerobic conditions. Bacterial suspensions were centrifuged at 6000 rpm for 10 min (R-10M, REMI, Mumbai, India), then the broth was discarded. The cellular

pellets were washed twice with sterile saline solution by a re-suspension step followed by centrifugation. Several cellular pellets were gathered together in order to obtain a bacterial *inoculum* of approximately 9 log CFU/ml. Thereafter, the *inoculum* was directly poured onto the substrate and homogeneously mixed in order to theoretically reach 8 log CFU/g of the rearing substrate. The actual bacterial concentrations of *inocula* were determined following the procedure reported below.

Enumeration of *L. monocytogenes* was carried out by spreading 0.1 ml of the ten-fold serial dilutions on ALOA plates (Agar Listeria Ottaviani Agosti, Biolife Italiana srl, Milan, Italy). The plates were incubated at 37 °C for 48 h. The results were expressed as log CFU per g or ml.

The mealworms were weighed in sterile stomacher bags and killed by freezing at -18 °C for 1 h; subsequently, the larvae were mixed with sterile saline solution, thoroughly cracked and homogenised for 60 s in a stomacher (Stomacher® 400 Circulator, VWR International Sr, Milan, Italy).

2.3. Technological treatments

Washing - Ten g of larvae were washed in 90 ml of sterile saline solution inside a sterile stomacher bag. This bag was thoroughly shaken for 3 min, then the washing solution was removed by pipetting and employed for microbial determination. The washed larvae were collected and used for microbial determination as well.

Fasting - Larvae were collected from the experimental boxes and submitted to a starvation process in sterile plastic containers with plastic web as base. Frass were collected in a second sterile plastic container placed below the plastic web.

Cooking - Larvae were cooked in a pre-heated oven at 150 °C for 10 min.

2.4. First trial

For the first trial, three different batches of larvae (500 g) were reared in sterile boxes. *L. monocytogenes* was added in a standardized concentration (see section 2.2.) to the batches in the boxes. Microbiological determinations were carried out on the larvae (washed and un-washed) and rearing substrates after 1, 2, 3 and 7 days (T1, T2, T3 and T7) from *L. monocytogenes* contamination. Persistence and enumeration of *L. monocytogenes* in the substrate (without larvae) was also carried out in the three contaminated boxes under the same experimental conditions.

Furthermore, microbiological determination was performed on the solution resulting from the larvae washing step. The experimental design of the first trial is represented in Figure 1.

2.5. Second trial

The experimental design of the second trial is represented in Figure 2. For the second trial, six different batches of larvae (500 g) were reared as previously described. At T7, each box was split into three sub-samples. No fasting, fasting for 24 or 48 h was applied to the three sub-samples, respectively. Two aliquots of each sub-samples were subsequently analysed as un-washed and washed larvae. Thereafter, the six types of interactions (non-fasted, fasted for 24 or 48 h - washed or un-washed) were oven cooked, for a total of twelve different combinations of treatments per batch (Figure 2). Enumeration of *L. monocytogenes* was performed for all the different types of samples as well on the washing solutions and collected faeces (frass).

2.6. Statistical determination

One-way ANOVA analysis was performed to evaluate the results from the first trial and to assess the effect of time (T1, T2, T3 and T7), while a Student T test was performed to assess the effect of washing (at fixed times). Similarly, the data concerning the substrates were analysed by a one-way ANOVA to assess the effect of time (T1, T2, T3 and T7) and a Student T test was performed to assess the effect of the larvae presence (only substrate *vs* rearing substrate, tested at fixed times). A two-way ANOVA test was employed to analyse results from the second trial with fasting and washing as main factors, interaction fasting × washing was also tested. The effect of cooking was tested via the Student T test between un-cooked and cooked samples within the washing and fasting effects. Statistical significance was set at 0.05 and differences were assessed using Tukey's test. R free statistical software was used (R Core Team, 2015).

3. Results and discussion

Following the proposed procedure, the amount of *L. monocytogenes* at the beginning of the trials was 8.24 ± 0.46 log CFU/g of substrate. *L. monocytogenes* did not affect the viability of mealworms since no mortality was noticed; furthermore, no alterations in morphology, behaviour and development were observed.

Table 1 shows the results obtained from the first trial concerning the enumeration of *L. monocytogenes* in the larvae, in the washed larvae, and in the substrate with and without larvae.

159 Statistical analysis revealed no significant effect of sampling time in both un-washed and washed
160 larvae. Furthermore, no significant effect was detected between cell concentrations detected
161 before and after the washing procedure. The data obtained from the enumeration of *L.*
162 *monocytogenes* in the washing solution (data not shown) did not reveal any difference in relation
163 to the sampling time, with an average concentration of 0.76 ± 1.34 log CFU/ml.
164 No data are available on the persistence of *L. monocytogenes* in mealworms reared in a
165 contaminated substrate. However, the data obtained from this research showed that if the
166 mealworms are accidentally contaminated by this bacterium, they can vehicle it. Furthermore, since
167 the washing step did not produce any advantages in terms of bacterial decontamination, it is
168 possible that most of the bacterial cells were housed inside the larvae. This hypothesis was also
169 supported by the low amount of *L. monocytogenes* found in the washing solutions.
170 *L. monocytogenes* was enumerated on the substrate without larvae and on the rearing substrate for
171 7 days. As reported in Table 1, time significantly affected the presence of *L. monocytogenes* in the
172 substrate without larvae, showing a decrease of the bacterial concentration between T1 and T7.
173 This result was partially expected, and in effect was seen in the lower *L. monocytogenes*
174 concentration at T1 than the theoretical amount.
175 On the other hand, substrate samples from the boxes with the larvae showed a quite stable *L.*
176 *monocytogenes* concentration between T1 and T7, with a mean value of 5.80 log CFU/g.
177 Results concerning the substrates revealed that the *L. monocytogenes* is able to survive in the
178 employed feed; nevertheless, the presence of the larvae contributes to maintaining the
179 contamination constant and this may be due to the digestion of the feed and ejection of the faeces
180 or to the steady aeration of the substrate. However, the presence of the larvae did not show any
181 statistically significant effect.
182 Recently, Wynants et al. (2019) tested the risk related to the presence of *Salmonella* spp. during
183 rearing of mealworms. *Salmonella* spp. showed to persist in the rearing substrate (wheat bran)
184 without larvae with no significant count variations.
185 On the other hand, different results occurred when larvae were present into the substrate. Wynants
186 et al. (2019) reported a significant decrease of *Salmonella* spp. amount (about 2.1 log CFU/g). These
187 findings highlight a different response of the bacteria both to the rearing substrates and to the
188 larvae presence, therefore a different risk of persistence.
189 Figure 3 shows the results from the second trial. The washing step did not affect the bacterial load
190 ($P = 0.320$), and neither did the interactions between fasting and washing ($P = 0.392$). Enumeration

of *L. monocytogenes* in the washing solutions showed an average concentration of 0.84 ± 1.52 log CFU/ml (data not shown).

Fasting affected the amount of *L. monocytogenes* with significant differences between un-fasted larvae and fasted larvae (reduction of ≈ 2 log CFU/g) ($P < 0.001$); no difference was detected between fasting for 24 or 48 h (Figure 3).

Other authors have reported that fasting and washing procedures did not affect the microbial loads, with minor differences linked to the evaluated microorganism and to the starvation or the washing methods employed (Wynants et al., 2018, 2017). As *L. monocytogenes* was never found in mealworms, no data are available on the effects of these procedures on this microorganism.

Cooking the larvae at 150 °C for 10 min was effective in killing *L. monocytogenes* cells, leading to the absence of the microorganism, whether or not the larvae were fasted and/or washed ($P < 0.001$). These results are in accordance with all the data available in the literature on the absence of *L. monocytogenes* in edible insect products sold as food and specifically in dried/cooked mealworms (Garofalo et al., 2017; Grabowski and Klein, 2017b, 2017c; Osimani et al., 2017).

Nonetheless Fasolato et al. (2018) reported to have isolated, via the MPN method, strains identified as *Listeria fleischmannii* in salted mealworms purchased from online commercial suppliers, however it seems that the tested ready-to-eat insects were unable to support the growth of the *Listeria monocytogenes* due to their low a_w.

Frass samples obtained for larvae fasted for 24 or 48 hours showed no statistical differences, with a mean value of *L. monocytogenes* load of 5.02 ± 0.15 log CFU/g of frass. The results were expected as frass is constituted by mealworms gut content and, due to its higher concentration in microbial loads and lower amount of moisture, it might contain a higher amount of *L. monocytogenes* than the whole body of the untreated mealworms.

For the trials, mealworms close to the pupation phase were employed, since this is the most frequently used stage for food and feed purposes; hence, several larvae turned into pupae during the trials. Consequently, the pupae were collected and then the samples were processed. As the pupae were randomly allocated into the different sampling times and after the different fasting periods the number of samples was insufficient for adequate analysis of the data, all the samples gathered nevertheless resulted to be negative for *L. monocytogenes*. These preliminary data could be very important as they might represent a starting point for studying microflora modification during the different life stages of mealworms. On the other hand, the absence of *L. monocytogenes* in mealworm pupae could be hypothesized *a priori,* since during the pupation process the larvae

completely purge the gut and moult, naturally decontaminating themselves internally and externally.

4. Conclusions

The data obtained in this study revealed that *L. monocytogenes* does not negatively affect the larvae viability; however, if present in the rearing substrate, it seems able to persist. *L. monocytogenes* concentration was not influenced by the washing procedure, while fasting was effective in significantly reducing its load. Nevertheless, the cooking process resulted to be a sound method for killing *L. monocytogenes* in *T. molitor* larvae reared in a contaminated substrate.

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341 Table 1. Results of the first trial on the enumeration of *Listeria monocytogenes* (log CFU/g) in larvae,

342 washed larvae, substrate without larvae and rearing substrate.

	Larvae				
	Time (T, days)				P - *Effect*
	T1	T2	T3	T7	*of time*
Larvae	3.64 ± 0.16	3.83 ± 0.85	4.06 ± 1.21	4.65 ± 0.47	0.215
Washed larvae	3.56 ± 0.10	4.11 ± 0.30	3.58 ± 0.66	4.11 ± 0.67	0.373
P - *Effect of washing*	0.499	0.616	0.580	0.133	

	Substrate				
	Time (T, days)				P - *Effect*
	T1	T2	T3	T7	*of time*
Substrate	6.58 ± 0.08[a]	6.02 ± 0.07[ab]	5.79 ± 0.87[ab]	4.86 ± 0.75[b]	0.013
Rearing substrate	6.57 ± 0.87	5.79 ± 0.53	6.10 ± 1.25	5.27 ± 0.79	0.235
P - *Effect of larvae presence*	0.995	0.506	0.740	0.372	

[a, b] in the same row indicate significant differences at P <0.05.

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Figure 1. Experimental design of the first trial.

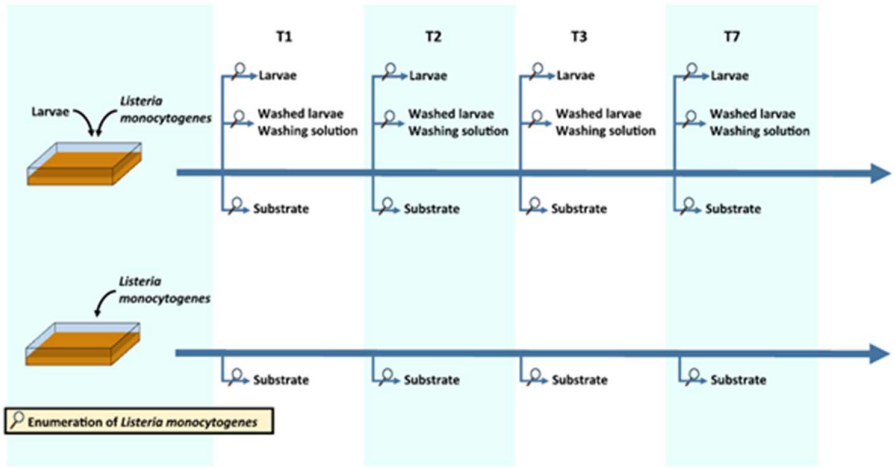


Figure 2. Experimental design of the second trial.

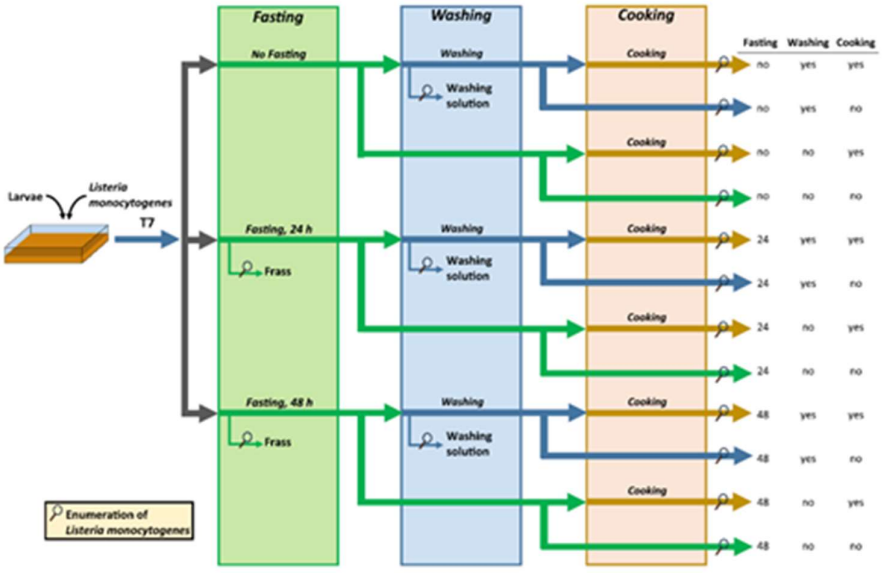
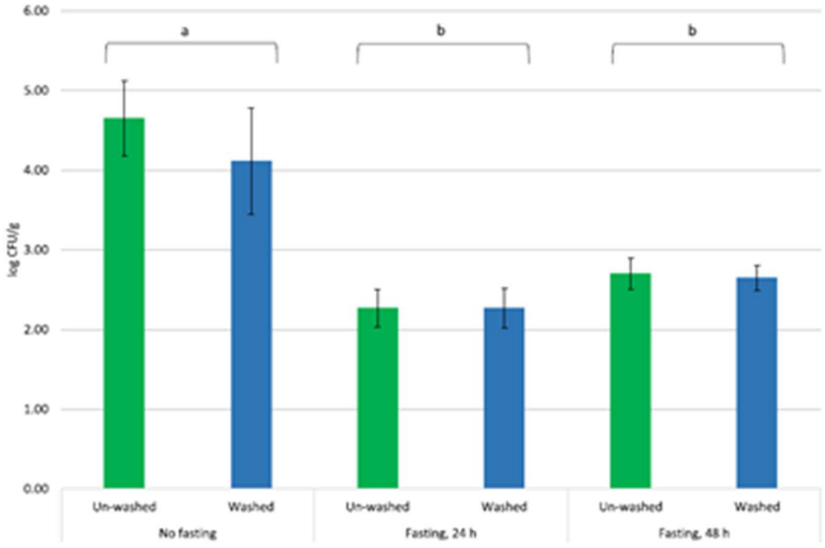


Figure 3. Results of the second trial, effects of fasting and washing on the enumeration of *Listeria monocytogenes* in *Tenebrio molitor* larvae.



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359 Figure 3 captions.

360 [a, b] indicate significant differences at P <0.05 for the fasting treatment. The standard deviations were

361 reported as error bars.