Animal 18 (2024) 101221

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Microbiological safety assessment of silkworm farms: a case study

L. Tassoni [a,*], S. Belluco [b], F. Marzoli [b], B. Contiero [a], S. Cremasco [c], A. Saviane [d], S. Cappellozza [d], A. Dalle Zotte [a]

[a] Department of Animal Medicine, Production and Health, MAPS, University of Padova, Agripolis, Viale dell'Università 16, 35020 Legnaro, Padova, Italy
[b] Laboratory of Safety and Quality of the Food Chain of Istituto Zooprofilattico Sperimentale delle Venezie, Vicenza, Italy
[c] Aulss 6 Euganea, Padova, Italy
[d] Centre of Research of Agriculture and Environment, Laboratory of Sericulture (CREA-AA), Padova, Italy

ARTICLE INFO

Article history:
Received 1 March 2024
Revised 4 June 2024
Accepted 13 June 2024
Available online 20 June 2024

Keywords:
Bombyx mori
Microbial load
Microbial safety
Rearing process
Silkworm pupa

ABSTRACT

Silkworms have been farmed for their silk since ancient times. After silk reeling, their chrysalides are consumed as food in several Asian countries. Despite the long rearing tradition of this insect, few studies have investigated the silkworm's microbiological safety all along the life cycle, focusing on detecting silkworm pathogens or on the safety of the dried chrysalis for food consumption. However, the in-farm rearing process, which takes around forty days, may affect the microbial load of the silkworm and of the rearing environment, as well as the quality of fresh cocoon and other performance parameters. No data is available on how microbial contamination changes during the rearing period and between different farmers. Furthermore, in light of the possible use of the chrysalis as food, it is crucial to understand how its microbial load varies according to the water content. To address these specific questions, we conducted an investigation involving the analysis of specific microbial indicators commonly used in the food chain. We collected environmental and silkworm samples from several farms. The examination covered the entire life cycle of silkworms, beginning with the first instar larvae and concluding with the scrutiny of both freshly harvested and dried pupae. Silkworm farms in Northeast Italy proved to be an appropriate model system for carrying out the experimentation. Additionally, an evaluation of rearing performance was conducted, with a focus on the quality of fresh cocoons and the survival rate of the insects.

Implications

The present study addresses the lack of data on changes in microbial contamination during rearing and among different farms. Furthermore, it emphasises the importance of understanding how microbial load varies during the process in view of the potential use of dried pupae as a novel food. The data obtained could be useful to farmers in implementing new safety measures and identifying critical points in their rearing practices. Finally, the rearing performance indicators, including the pupal survival rate, are essential indicators for the silk industry and for evaluating farmers' performances.

Introduction

Several insects are considered to be viable and sustainable sources of protein suitable for both human and animal consumption. Their traditional use as food has been widely documented in literature for Asian countries (Mitsuhashi, 1997; Yhoung-Aree et al., 1997; Mishra et al., 2003; Han et al., 2017). However, over the last decade, insect-based products have also gained popularity in Western countries. Regulations aimed at ensuring consumer safety have therefore been gradually applied to their production (EFSA Scientific Committee, 2015). In countries with a tradition of insect consumption, wild harvesting is common; conversely, Western countries have adopted an intensive farming approach to ensure stable and efficient insect production all over the year. This is the case for many insects authorised as feed ingredients or evaluated as a novel food in Europe (EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) et al., 2021b; 'Commission Regulation (EU) 2017/893 of 24 May 2017; 'Commission Regulation (EU) 2021/1925 of 5 November 2021).

Unlike other insects, which have been exploited for mass rearing only in the 21st century, the silkworm (*Bombyx mori*) was introduced to the Byzantine Empire in the 6th century AD according to the legend (Lecocq, 2019). Furthermore, in Europe, sericulture is predominantly carried out by small farmers due to the absence of automation and the restricted vegetative season of mulberry, which is the sole food source for *Bombyx mori*.

* Corresponding author.
E-mail address: luca.tassoni@phd.unipd.it (L. Tassoni).

https://doi.org/10.1016/j.animal.2024.101221
1751-7311/

Since silkworms are farmed animals and could potentially be used as feed ingredients or food in the future ('Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015), it is important to investigate and monitor the microbiology of both the rearing environment and the animal itself. Currently, there are available data on the safety of the silkworm pupae as food (Marzoli et al., 2022). However, to our knowledge, no specific research has systematically examined the microbiology of silkworms throughout their whole life cycle in current farming systems, excluding studies solely focused on harmful microorganisms causing diseases in the insect (Pasteur, 1870). This study examines microbial contamination of silkworm larvae and their rearing environment during the various phases of the rearing process in the Veneto region (Italy) in the spring season of 2022.

We wanted to investigate whether the microbial load of silkworms at different life stages could be significantly different and whether it was influenced by different rearing practices, although they originated from the same larval source. We also wanted to assess whether current rearing and processing techniques allow the production of fresh and dried pupae with microbiological qualities that meet the standards required for their use in the food and feed sectors.

The study focuses on microorganisms that are relevant for food and feed safety, as well as microorganisms commonly used as hygiene indicators to determine if they can be used to monitor good practices in the farming system. Additionally, we explored potential correlations between the microbiological indicators and environmental variables, such as temperature and relative humidity.

Material and methods

Selection of the silkworm farms and hygiene protocol

This study monitored five silkworm farms in the Veneto region (Northeast Italy). The farms were selected based on the farmers' rearing experience and compliance with silkworm-rearing guidelines prepared by the Centre of Research of Agriculture and Environment, laboratory of Sericulture (**CREA-AA**) (Cappellozza, 2010) (Supplementary Figure S1). This public research institution assists silkworm farmers and produces pathogen-free silkworm eggs. In this experimental study, CREA-AA provided young larvae of the same polyhybrid strain to all participating farms. Due to the ongoing collaboration, CREA-AA researchers were able to easily monitor the farms, resulting in accurate information collection on the effective specific rearing practices adopted by farmers, including the implemented safety measures.

Following the production protocol mentioned above, farmers are required to sanitise all tools and rearing facilities before use. Additionally, they must identify and maintain optimal environmental conditions throughout each phase of rearing. The protocol essentially conveys the disinfection rules outlined in the Food and Agriculture Organization manual (Pang-chuan and Da-chuang, 1988) to Italian farmers clearly and understandably. Hydrogen peroxide was suggested as a preferable disinfectant, to replace formalin in environmental sanitation due to its minimal environmental footprint and the absence of hazardous residues for human safety. Furthermore, hydrated lime can be used for bed cleaning and a diluted solution of sodium hypochlorite can be employed to sanitise instruments and facilities. These practices aim to minimise the risk of silkworm diseases and obtain high-quality fresh cocoons in the context of sustainable agriculture.

Silkworm rearing and cocoon processing

Silkworm rearing is typically organised in a centralised manner wherein a nursery farm is responsible for growing the silkworms until the third instar. After this phase, the young larvae are distributed to farmers to complete the rearing process for commercial cocoon production. Collective incubation and rearing of the young larvae by experienced workers are employed to mitigate rearing errors during a period when silkworms are particularly susceptible to diseases. In the case study described in the paper, only one farmer reared silkworm larvae from the first instar to the cocoon spinning stage, while others received third-instar larvae. It is noteworthy that the handling procedures differ between the initial three instars and the subsequent two. In the first three instars, larvae are fed cut leaves. This process requires daily removal of litter, which involves extensive handling by operators. However, during the last instars, larvae feed directly on the leaves of the mulberry branches. These branches are administered at regular intervals, forming a layered structure. Larvae ascend to consume fresh leaves, while the dried branches allow litter to fall through the gaps (Fig. 1).

Mulberry leaves and branches are typically sourced from the mulberry fields surrounding the farm. Farmers monitor and partially control temperature and relative humidity using stoves and fans to compensate for external environmental fluctuations, following the environmental parameters suggested in the rearing manual (Pang-chuan and Da-chuang, 1988). All farmers conducted a sanitising treatment before receiving the larvae, and each farmer implemented different sanitising procedures and organised the farming environments in a specific way, as summarised in Table 1.

The farmers were organised as shown in Fig. 2. Farm 0 and Farm 1 received the newly hatched larvae, at the first instar, from the egg incubation centre (CREA-AA). Farm 1 reared the larvae until the cocoon was completed, while Farm 0 (nursery) reared the larvae until the beginning of the third instar, keeping them separated in batches of 20 000 larvae (silkworm boxes). Subsequently, Farm 0 allocated the predetermined number of larvae to Farm 2, Farm 3, and Farm 4. Farm 2, Farm 3, and Farm 4 reared the larvae from the third instar until cocoon completion.

After completing the cocoon stage, all farmers (Farm 1 to Farm 4) deflossed the cocoons (silk floss removal from cocoons) and sorted them into first and second quality. The main differences between first and second−quality cocoons lie in the occurrence of shape irregularities or minor stains in the latter. The viability of fresh pupae remains consistent between first and second−quality cocoons. First−quality cocoons undergo drying followed by boiling during the reeling process, whereas second−quality cocoons are solely dried to prevent pupal metamorphosis and their silk is used for innovative applications that do not require reeling. Usually, these cocoons are cut and the chrysalides are extracted to separately use fibroin and sericin of the silk shell. Consequently, pupae from second−quality cocoons are optimal candidates for use in the food chain. The cocoons were then placed into jute bags, according to their batch number, and delivered to CREA-AA. At this point, the cocoons were dried in a ventilated oven at 80 °C for 6 h, followed by 2 h at 70 °C and 2 h at 60 °C.

The number of reared larvae differed among farms. Farm 2 and Farm 3 each reared 40 000 larvae divided into two batches. Farm 4 reared 80 000 larvae, divided into four batches. Farm 1 reared 140 000 larvae divided into seven batches, with each batch corresponding to a box of silkworm eggs. These batches were physically separated and traceable from hatching until cocoon drying.

Experimental sampling

The purpose of this study was to analyse the microbiological contamination of both silkworms and their rearing environments. Samples were collected at various life stages of the silkworms, including: at the beginning of the third instar, in the middle of the fifth instar (fifth day), during spinning (prepupa), in the pupal

Fig. 1. Difference in silkworm rearing methods between the first three instars (on the left) and the last two instars (on the right).

Table 1
Features of the rearing environments and sanitising procedures adopted by each silkworm farm.

Farmer	Environment sanitising method	Tools sanitising method	Reared larval instars	Rearing facility organisation	Notes
0	H_2O_2 (35%) sprayed throughout the rearing room	Dipping in a diluted solution of sodium hypochlorite (0.5%) for 24 hrs; water rinsing, drying under sunlight	1st to 3rd (newly hatched larvae received from CREA-AA, Padova, Italy)	one single room used for shelf-rearing from 1st to 3rd instars	Shoe- sanitising procedure, Gloves and overcoats used by operators at litter changes
1	H_2O_2 (35%) sprayed throughout the rearing room	H_2O_2 (35%)	1st to cocoon (1st instar larvae received from CREA-AA, Padova, Italy)	one room for the first 2 instars, a wider or larger room from the 3 rd instar to cocoon spinning	Shoe-sanitising procedure
2	Hydrated lime	H_2O_2 (35%)	3rd to cocoon spinning (3rd instar larvae received from the nursery farm)	one single room from 3 rd instar to cocoon spinning	No shoe-sanitising procedure
3	High-pressure washer at high temperature (140–150 °C)	Dipping in a diluted solution of sodium hypochlorite (0.5%) for 24 hrs	3rd to cocoon spinning (3rd instar larvae received from the nursery farm)	one single room for instars from 3 rd to cocoon, increasing the dedicated space (reducing density) as rearing progresses	No shoe- sanitising procedure; dogs allowed to occasionally enter the room
4	High-pressure washer with water and bleach in the first room, hydrated lime in the second room	High-pressure washer with water and bleach	3rd to cocoon (3rd instar larvae received from the nursery farm)	one room for the 3 rd and 4th instars, and a wider room from the 5th instar to cocoon spinning	Overshoes only to enter the first room; the second room also hosts two cows

stage (fresh pupa) and the pupal stage after cocoon drying (dried pupa) (Fig. 3). Each sample consisted of approximately 50 g of fresh larvae or pupae individually selected from a limited area of the rearing rooms. For each life stage mentioned above, three independent samples were randomly collected, from distinct positions within each farm.

Environmental samples were collected from a 900 cm^2 surface using an environmental sponge. Three different areas were sampled inside each farm for both insect and environmental samples. The samples were then cold stored at 4 °C and transported to the analysis lab within 3 h of the collection. The sampled area consists of the surface at the bottom of the rearing area, where leaves and branches were placed. This area in the last instars was not in direct contact with silkworms but only with their litter.

Throughout the experimental period, one operator was solely responsible for collecting all samples and took appropriate measures to prevent contamination of both the surroundings and the insects, as well as to avoid cross-contamination of the experimental material. The insects collected were consistently taken from the same rearing batches within each farm. The operator responsible for collection wore alcohol-sterilised gloves and a face mask while handling the sampling materials. Samples of larvae and fresh pupae were collected on-site directly from the farms, while dried pupae were obtained from CREA-AA where they were dried shortly after the delivery by the farmers.

Both fresh and dried pupae were extracted from cocoons by cutting them. Cutting instruments were disinfected when moving from one repetition or batch to another. Sensors were employed to monitor the temperature and humidity of farms 1, 2, 3, and 4 from the fifth instar up to the spinning phase. Mean, minimum and maximum values of temperature and humidity were calculated daily based on gathered data.

Microbiological and statistical analyses

Microbiological analyses were performed on both the silkworm and the environmental samples based on the advice of a public authority veterinarian. The analyses aimed to detect a wide variety of microbes using standard procedures that can be implemented in possible hygiene self-monitoring procedures. The following analyses were performed on each sample: enumeration of presumptive *Bacillus cereus* at 30 °C, enumeration of moulds and yeasts, mesophiles count (total viable count at 30 °C), and enumeration of coliforms at 30 °C.

Bacillus cereus was included among the indicators due to its previous documentation in studies concerning silkworms (Li et al., 2015; Fasolato et al., 2018; Frentzel et al., 2022). *Bacillus cereus* is a foodborne pathogen that can survive in harsh environments by producing spores and biofilms. It represents a serious food safety issue due to its ability to synthesise various toxins that cause gas-

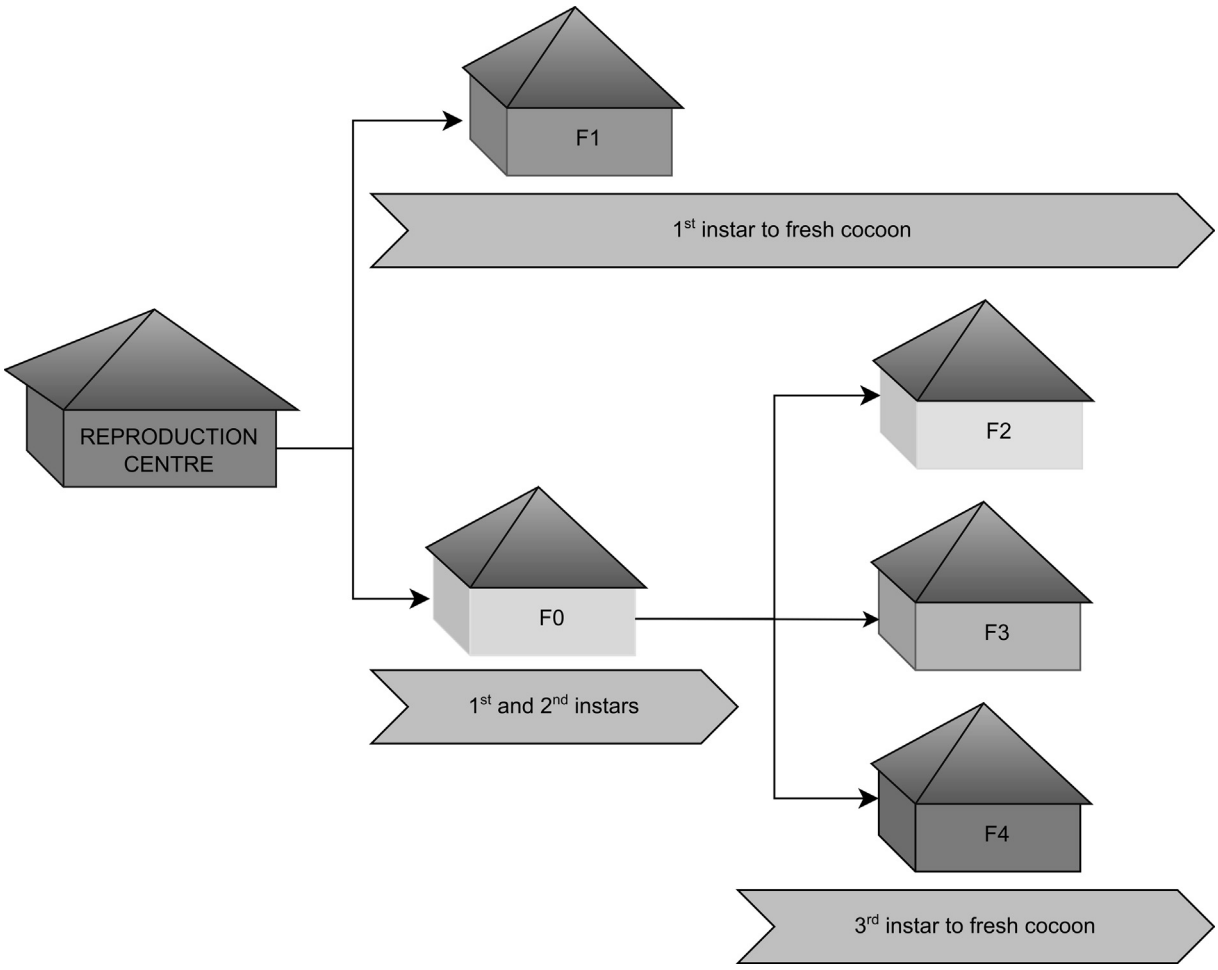

Fig. 2. Scheme of the experimental silkworm rearing organisation. The reproduction centre (CREA-AA, Padova, Italy) provided newly hatched larvae to Farm 0 (F0) and Farm 1 (F1). Farm 0 reared the larvae until the beginning of the third instar and then allocated them to Farm 2, Farm 3 and Farm 4 (F2, F3, F4), which reared them from the third instar to cocoon completion. On the other hand, Farm 1 reared the larvae from the first instar until cocoon completion.

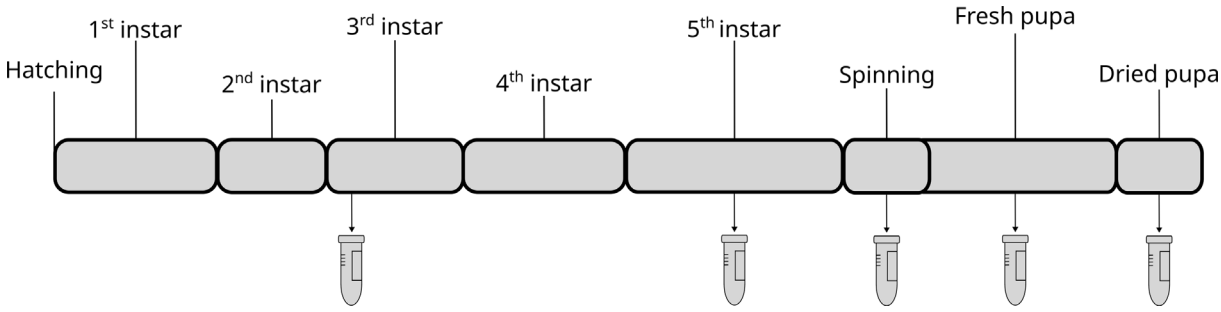

Fig. 3. Silkworm sampling scheme. Sampling was designed in relation to the silkworm life stages and processing steps. All samples were collected alive on the farm except for the dried pupae collected at CREA-AA. Displayed lengths were proportional to each life stage duration.

trointestinal diseases (Huang et al., 2020). We did not search for specific pathogens like *Salmonella* spp. due to the lack of literature evidence indicating their presence in silkworms (Marzoli et al., 2022).

Sample processing of silkworms and environmental sponges was conducted according to ISO 6887:2017 and ISO 18593:2018. Briefly, 20 g of silkworm sample was diluted in buffered pepton water (**BPW**) in 1:10 ratio. Regarding environmental sponges, BPW was added in relation to the sampled surface allowing the expression of results as colony forming unit per cm^2 (CFU/cm^2). After BPW addition, all the samples were thoroughly homogenised and diluted.

The references for the methods for microorganism quantification and their limit of detection are reported in Supplementary Table S1.

These microorganisms are considered generic indicators of hygiene in the food production chain. They could also be used to evaluate good hygiene practices in the future production of silkworms for food purposes. The analyses were conducted at the Laboratory of Safety and Quality of the Food Chain of Istituto

Zooprofilattico Sperimentale delle Venezie (Vicenza, Italy) (an Italian health authority and research organisation in the fields of animal health, food safety and zoonoses).

The data were analysed using SAS software, version 9.4 (Copyright © 2002–2012 by SAS Institute Inc., Cary. NC. USA) and XLSTAT for Excel (https://www.xlstat.com). Microbial count data were log-transformed (lg (x + 1)) to normalise the distribution. The normality of the log-transformed data was tested through the Shapiro-Wilk test. Factorial ANOVA was used to test the effect of farm and life stages on normally distributed data. The Kruskal-Wallis non-parametric test was used when normality was not achieved. Posthoc pairwise comparisons among levels were performed using Tukey's or Dunn's correction. Spearman's rank correlation was used to test the potential relationship between different microbial indicators, as well as the microbial counts and environmental parameters, i.e. humidity and temperature values.

Rearing performance parameters

Silkworms are primarily reared for silk production. Therefore, we collected parameters related to the cocoons obtained at the end of the process. Specifically, we measured the total fresh weight of cocoons produced by each farmer. The cocoons were then sorted based on the quality (Vasta et al., 2023), and we estimated the quantity of first-quality cocoons. These data were recorded when the farmers delivered the fresh cocoons they produced to CREA-AA for drying.

The survival rate of silkworm larvae was also estimated by calculating the ratio between the final number of fresh cocoons obtained by each farmer and the precise number of larvae they initially received as presented in Equation 1. A representative sample was randomly collected from each farmer to assess the average weight of fresh cocoons (**FC**) and the total fresh cocoon weight (**TFC**) was used to estimate the number of fresh cocoons. The total number of initial larvae was estimated by multiplying the initial number of batches (**ILB**) per the number of larvae per box (**B**).

$$\text{Estimated percentage of larvae reaching the pupal stage} = (\text{TFC}/\text{FC})/(\text{ILB} \times \text{B})$$

The survival parameter is crucial in assessing rearing performance, as a higher survival rate of the larvae is correlated with an increased yield of fresh cocoons. Additionally, it plays a vital role in addressing concerns related to animal welfare. It is anticipated that the consideration of animal welfare in insect production will gain increasing influence in the coming years (Pali-Schöll et al., 2019; Delvendahl et al., 2022; Klobučar and Fisher, 2023; 'IPIFF Ensuring High Standards of Animal Welfare in Insect Production, 2019').

Results

Silkworm microbiological data

The microbiological data exhibited significant variability, with even the means obtained from triplicate analyses of the same farm occasionally displaying high SDs. These deviations indicate a substantial variation in microbiological contamination within the same room.

The coliform counts exhibited a wide range, varying from < 1.04 lg CFU/g (detected in dried pupae of Farm 1 and Farm 2) to 7.34 lg CFU/g, which was found in third-instar larvae reared in Farm 0. Notably, high coliform counts were also observed in the spinning larvae of Farm 4 and the fresh pupae of Farm 3, reaching 7.23 lg CFU/g and 7.11 lg CFU/g, respectively. For dried pupae, coliform values ranged from < 1.04 lg CFU/g to 3.58 lg CFU/g. The highest value was exhibited by Farm 3, while Farms 2 and 1 displayed the lowest value of < 1.04 lg CFU/g (Table 2).

Mesophile counts ranged from 2.38 lg CFU/g to 8.04 lg CFU/g, with the lowest value observed in dried pupae and the highest in third-instar larvae. Fresh pupae displayed mesophile counts ranging from 4.18 lg CFU/g to 7.56 lg CFU/g, both values derived from two samples collected at the same farm (Table 2).

The mould counts exhibited a range from < 0.3 lg CFU/g to 5.76 lg CFU/g. None of the fresh pupal samples exceeded the value of 3.91 lg CFU/g, which was recorded at Farm 1. Moulds were not detected in the dried pupae from Farm 2 and Farm 4. However, one sample from Farm 3 and one from Farm 1 were contaminated with moulds, with counts of 2.7 lg CFU/g and 4.93 lg CFU/g, respectively (Table 2).

Yeasts were below the limit of detection in the majority of the samples. The highest yeast counts were measured in third-instar larvae from Farm 0, reaching 5.58 lg CFU/g. Notably, samples of spinning larvae and fresh pupae showed no yeast contamination. However, fifth-instar larvae from all the farms showed yeast contamination, with counts equal to or above 2.30 lg CFU/g. In the case of dried pupae, yeasts were below the limit of detection (< 2 lg CFU/g) except for two samples collected in Farm 2 reaching 2.6 lg CFU/g and one collected in Farm 3, which reached 3.49 lg CFU/g (Table 2).

Presumptive *Bacillus cereus* was absent from all but one of the larval samples except one obtained from Farm 4 during the fifth instar. No pupal samples, either fresh or dried, displayed any detectable contamination of *Bacillus cereus*.

The boxplots displaying the values of the three replicates are reported in Supplementary Figures S2–S5.

ANOVA was employed to test for differences in coliforms and mesophiles among various stages and conditions. In terms of coliform counts, dried pupae displayed the lowest value, and the difference with other samples was significant. Spinning larvae and fresh pupae showed significantly higher counts compared to dried pupae and fifth instar larvae (Fig. 4a).

Similarly, the mesophile count was found to be the lowest in dried pupae, and this difference was statistically significant compared to the fifth instar, spinning larvae, and fresh pupae (Fig. 4b).

To assess differences in mould and yeast among life stages, the Kruskal-Wallis test was used due to the non-normal distribution of the sampling data. Mould microbial counts were significantly higher in the fifth instar compared to the spinning larvae, fresh pupae, and dried pupae (Fig. 5a). No significant differences were found among other life stages.

Yeast contamination levels also followed a similar pattern to moulds. Specifically, the fifth instar displayed significantly higher counts compared to spinning larvae, fresh pupae and dried pupae (Fig. 5b).

Differences among farmers were tested using Kruskal-Wallis test. Few significant differences were detected especially between F0 and F1 for coliforms, mesophiles and yeasts at the third instar (Supplementary Table S2).

Environmental microbiological data

Considerable variability was observed among farmers in the data obtained from environmental samples. The initial coliform values before starting silkworm rearing ranged from <0.3 to 3.94 lg CFU/cm^2, with Farm 1 exhibiting the highest count. The environmental microbial count of Farm 1 notably decreased during the third instar, while all farms experienced an increase in microbial count during the spinning phase. The coliform counts ranged from 4.17 to 4.92 lg CFU/cm^2 (Table 3). This increase in microbial count between the prerearing and spinning phases is a consistent trend across all farms, except for Farm 1. Before the start of rearing activ-

Table 2
Coliform, mesophile, mould and yeast counts (lg CFU/g) per farm and sampling moment in the silkworm rearing. The mean values of the three replicates were reported together with their SD.

Microorganism	Farmer	Third instar	Fifth instar	Spinning	Fresh pupa	Dried pupa
Coliforms	F0	7.2 (±0.3)	na	na	na	Na
	F1	5.4 (±0.2)	3.5 (±0.3)	4.9 (±1.1)	3 (±1.4)	0.7 (±1.1)
	F2	na	2.9 (±0.7)	5 (±0)	5.1 (±0.5)	0 (±0)
	F3	na	2.5 (±0.7)	5 (±0)	4.9 (±2.2)	2.8 (±0.9)
	F4	na	3.5 (±0.3)	5.7 (±1.5)	4.5 (±0.6)	2 (±0.8)
Mesophiles	F0	7.8 (±0.2)	na	na	na	Na
	F1	6.6 (±0.2)	5.4 (±0.1)	6 (±0.5)	5.1 (±0.2)	3.4 (±1.2)
	F2	na	5.2 (±0.3)	5.7 (±0.2)	5.6 (±0.2)	3.4 (±0.9)
	F3	na	5.2 (±0.3)	6.4 (±0.4)	5.6 (±1.8)	3.7 (±1.3)
	F4	na	5.9 (±0.2)	6.1 (±1.4)	5.3 (±0.2)	3.6 (±0.4)
Moulds	F0	3.9 (±0.8)	na	na	na	Na
	F1	3.8 (±0.5)	3.9 (±0.7)	0.9 (±1.5)	2.3 (±2)	1.6 (±2.8)
	F2	na	4.2 (±1.3)	1.6 (±1.4)	1.6 (±1.4)	0 (±0)
	F3	na	3.2 (±0.1)	2.3 (±0.4)	0.8 (±1.4)	0.9 (±1.6)
	F4	na	3.3 (±0.1)	0 (±0)	1.3 (±1.2)	0 (±0)
Yeasts	F0	4.2 (±1.3)	na	na	na	Na
	F1	0 (±0)	0.8 (±1.3)	0.7 (±1.2)	0 (±0)	0 (±0)
	F2	na	2.6 (±0.2)	0 (±0)	0 (±0)	1.5 (±1.4)
	F3	na	2.5 (±0.2)	0 (±0)	0 (±0)	1.2 (±2)
	F4	na	3.5 (±0.1)	0 (±0)	0 (±0)	0 (±0)

Abbreviations: na = Not applicable.

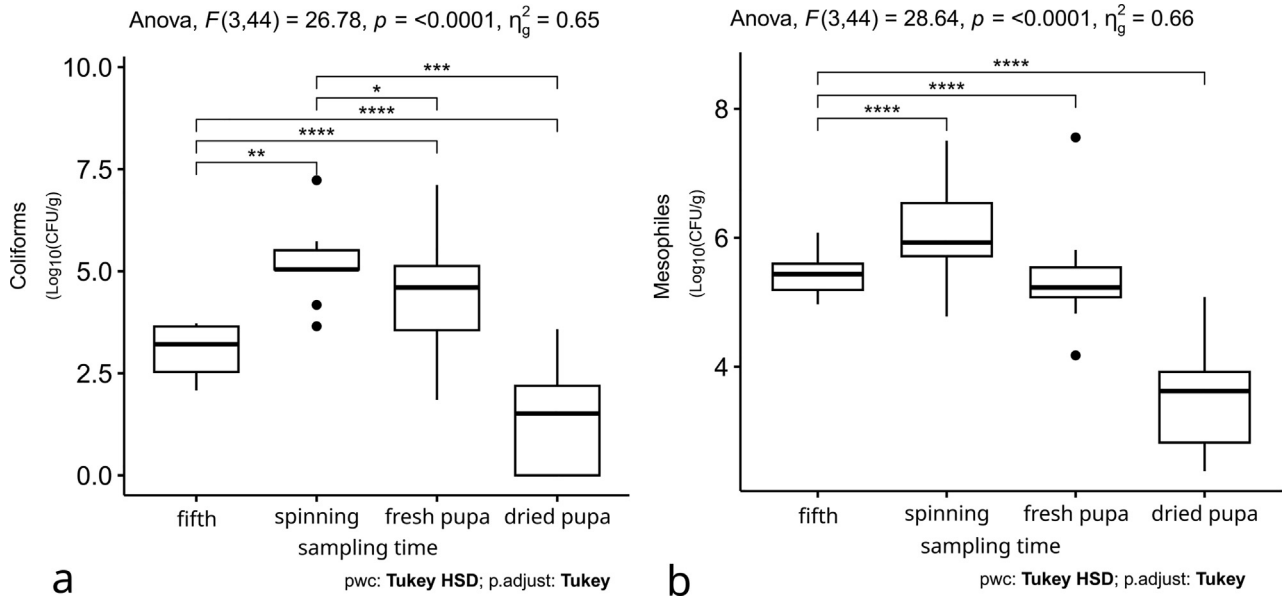

Fig. 4. Box plot charts reporting the coliform (a) and mesophile (b) microbial counts of fifth instar, spinning phase, fresh pupa and dried pupa samples of silkworm. A one-way ANOVA test was performed to identify significant differences, followed by TUKEY as posthoc to determine which life stages were significantly different. The *P*-values of TUKEY test were * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001. η^2_g = generalised eta squared; pwc = pairwise comparison; Tukey HSD = Tukey honestly significant difference test; p.adjust = adjusted *P*-value.

ities, mesophiles ranged from 0.77 to 6.14 lg CFU/cm^2 across all farms. During the spinning phase, mesophiles ranged between 3.04 and 5.92 lg CFU/cm^2, with Farm 3 exhibiting the highest counts (Table 3).

Moulds were not detected in Farm 0 and Farm 4 before rearing. However, a count of 4.83 lg CFU/cm^2 was found in Farm 2. The highest mould counts during the spinning phase were observed in Farm 1, ranging from 4.81 to 5.30 lg CFU/cm^2 (Table 3). At the beginning of the rearing process, Farms 0, 1, and 2 had no yeasts present in their environments, while Farms 3 and 4 had yeast counts ranging from 1.04 to 2.00 lg CFU/cm^2. During the spinning phase, yeasts were not detected in any of the farms except for Farm 4, where a sample showed a count of 2.23 lg CFU/cm^2 (Table 3). The boxplots displaying the values of the three replicates are reported in Supplementary Figures S6–S9.

Differences among farmers were tested using Kruskal-Wallis test and few significant differences were detected especially in the third phase as it regards mesophiles and moulds count (Supplementary Table S3).

Correlation between temperature and silkworm microbiological data

Table 4 shows significant findings from Spearman's correlation between larval microbiological counts and environmental conditions. The results indicate a positive correlation (correlation coefficient: 0.541, *P*-value: 0.004) between mean temperatures and

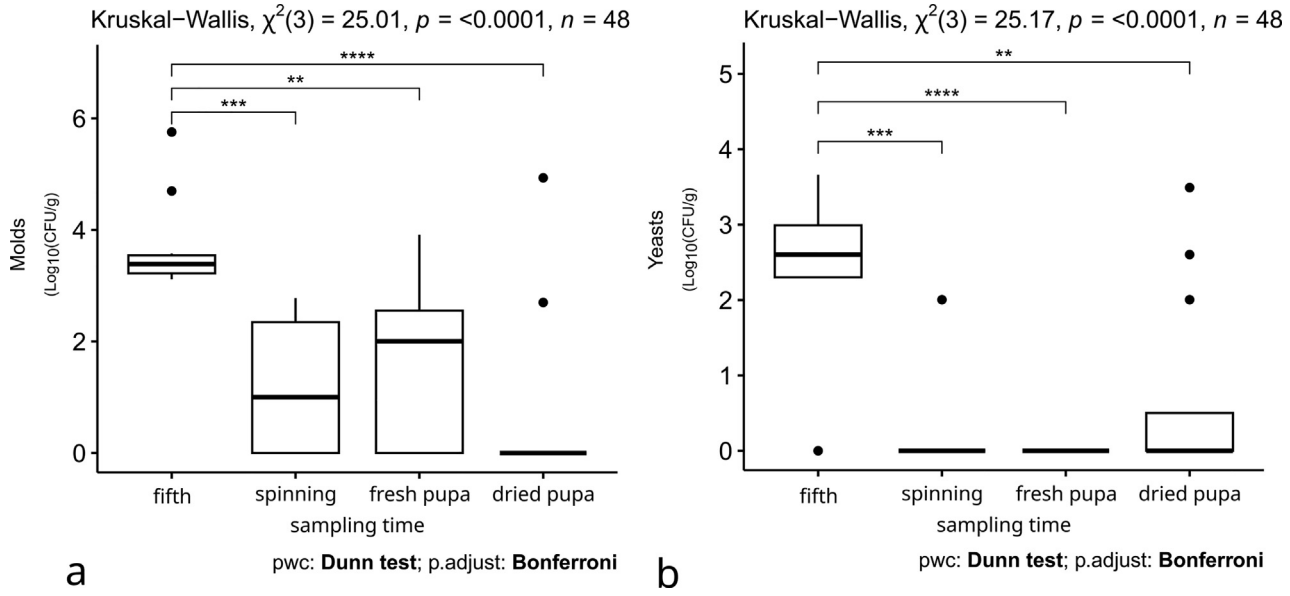

Fig. 5. Box plot charts reporting the mould (a) and yeast (b) microbial counts of fifth instar, spinning phase, fresh pupa and dried pupa samples of silkworm. A Kruskal-Wallis test was performed to identify significant differences, followed by Dunn's test to determine which instars showed significant differences. The *P*-values of Dunn's test were ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001. pwc = pairwise comparison; p.adjust = adjusted *P*-value.

Table 3
Coliforms, mesophiles, moulds and yeasts count (lg CFU/cm^2) per farm and sampling moment in the silkworm rearing. The mean values of the three replicates were reported together with their SD.

Microorganism	Farmer	Prerearing	Third instar	Spinning
Coliforms	F0	0 (±0)	0.4 (±0.4)	na
	F1	2.4 (±2.1)	1.1 (±0.9)	2 (±1)
	F2	0 (±0)	na	4.4 (±1.9)
	F3	1.6 (±1.4)	na	5.8 (±0.5)
	F4	0.4 (±0.6)	na	3.4 (±1.6)
Mesophiles	F0	2.4 (±0.3)	0.9 (±0.3)	na
	F1	5.4 (±1.1)	3.4 (±1.6)	3.9 (±0.2)
	F2	3.6 (±0.2)	na	4.8 (±0.4)
	F3	5.1 (±0.5)	na	5.8 (±0.2)
	F4	1.1 (±0.3)	na	4 (±1)
Moulds	F0	0 (±0)	1.7 (±0.8)	na
	F1	3.9 (±0.8)	0 (±0)	5.1 (±0.2)
	F2	2.3 (±2.4)	na	4 (±0.2)
	F3	4.2 (±0.2)	na	4.1 (±0.3)
	F4	0 (±0)	na	2.3 (±0.8)
Yeasts	F0	0 (±0)	0.3 (±0.6)	na
	F1	0 (±0)	0.4 (±0.8)	0 (±0)
	F2	0 (±0)	na	0 (±0)
	F3	0.7 (±1.2)	na	0 (±0)
	F4	0.3 (±0.6)	na	0.4 (±0.8)

Abbreviations: na = not applicable.

coliform counts, suggesting that higher temperatures were associated with increased coliform counts. Similarly, mesophiles correlate with mean temperatures (correlation coefficient: 0.410, *P*-value: 0.035). In contrast, moulds and yeast exhibit a negative correlation with mean temperature values (correlation coefficients of −0.418 and −0.680 and *P*-values of 0.031 and < 0.001, respectively).

Production performance indicators

The survival rates of the pupae varied among the selected farmers. Farmer 2 achieved the highest rate at 72%, closely followed by Farmer 4 with a rate of 71%. Farmers 1 and 3 recorded relatively lower survival rates, approximately around 50% (Table 5).

In terms of cocoon yield, Farmers 2 and 4 achieved the highest production of fresh cocoons per box compared to Farmers 1 and 3. The quantity of fresh cocoon per box was the highest for Farmer 4 at 33.9 kg, followed by Farmer 2 at 30.2 kg. Farmer 1 obtained 27.8 kg, and Farmer 3 obtained 26.9 kg. When assessing the production in relation to the quantity of first-quality fresh cocoons, the disparities among the farmers became more evident. Farmers 2 and 4 produced the highest percentage – and the highest quantity – of first-quality fresh cocoons compared to Farmers 1 and 3. Farmer 2 achieved the highest percentage of 65.6% (equivalent to 19.7 kg), indicating that a larger proportion of cocoons met the standards for the first quality. Farmer 4 obtained a percentage of 47.0%, while Farmer 1 and Farmer 3 had 50.2 and 21.0%, respectively (Table 5).

Table 4
Spearman's correlation matrix between silkworm larvae microbial counts and monitored temperature and humidity values.

Variables	Mean temp	Min temp	Max temp	Mean hum	Min hum	Max hum
Col_lv	541*	85	431*	93	−125	−142
Mes_lv	410*	201	321	43	−228	−374
Mol_lv	−418*	44	−390*	136	290	143
Yea_lv	−680*	−241	−430*	−147	30	−47

Abbreviations: Mean temp = Mean temperature in the rearing period; Min temp = Minimum temperature in the rearing period; Max temp = Maximum temperature in the rearing period; Mean hum = Mean humidity in the rearing period; Min hum = Minimum humidity in the rearing period; Max hum = Maximum humidity in the rearing period; Col_lv = Coliform count in larval phases; Mes_lv = Mesophile count in larval phases; Mol_lv = Mould count in larval phases; Yea_lv = Yeast count in larval phases.
* Values differ significantly from 0 at $P < 0.05$.

Table 5
Production performance indicators of the four farms. Each batch corresponds to a box of 20 000 silkworm larvae. The number of silkworm pupae per batch at the end of rearing was estimated based on the mean fresh cocoon weight. The survival rate was calculated by dividing the number of pupae per batch by the initial number of 20 000 larvae.

Farmer	n° of rearing batches	n° pupae/batch (estimate)	Survival rate (%)	Kg of fresh cocoons/batch	Kg of first-quality fresh cocoon/batch	Percentage of first-quality fresh cocoon/batch
F1	7	10 054	0.5	27.8	13.9	50.2%
F2	2	14 470	0.72	30.2	19.7	65.6%
F3	2	10 227	0.51	26.9	5.6	21.0%
F4	4	14 140	0.71	33.9	15.9	47.0%

In summary, Farmers 2 and 4 had the highest success rates in terms of pupal survival, yield of fresh cocoons, and quantity of first-quality cocoons. Farmer 1 demonstrated moderate results across most aspects, while Farmer 3 had the lowest performance, with the lowest survival rates and the lowest yield of first-quality cocoons.

Discussion

Silkworm microbiological data

Few statistically significant differences were identified when comparing the microbial counts of the same developmental stage among various farmers. The observed highest microbial counts in Farm 0 could potentially be attributed to the larger number of larvae reared in that farm in the current rearing organisation. High-density farming environments are inherently vulnerable to potential risk factors. The proximity of animals and the involvement of the same operators handling various batches contribute to the transmission of microorganisms among the animals. Additionally, ensuring adequate ventilation and frass removal in densely populated farms can be challenging, further facilitating the spread of microbes.

These differences, observed during the third instar, were lost after the larvae from Farm 0 were distributed to Farms 2, 3, and 4, where only a limited number of batches were reared at the same time. The comparative analysis of the microbial counts in fresh and dried pupae demonstrates the effectiveness of the drying process in significantly reducing the microbial counts of coliforms, mesophiles, and moulds. However, no differences were observed in yeast counts between fresh and dried pupae. This is not a relevant issue since the majority of both fresh and dried pupae samples showed no yeast contamination. Furthermore, it is reassuring to note that presumptive *Bacillus cereus* counts were below the detection level in all the samples. This alleviates concerns regarding this specific microorganism. Although there are no defined microbiological criteria for silkworms intended for human consumption, the present results can be compared with the limits reported in available scientific opinions available from EFSA (EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) et al., 2021a, b; EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA Panel) et al., 2023).

Upon examining the microbial counts in dried pupae, it is evident that most samples were free of moulds and yeasts. However, two samples displayed moulds count exceeding 2 lg CFU/g and 3 samples displayed yeast count equal to or exceeding 2 lg CFU/g. These values may be attributed to a high microbial count in the fresh pupae prior to the drying process or to errors in the management of the drying process. The approved levels for yeast and mould counts in already approved insect-based novel foods are 2 lg CFU/g (100 cfu/g) for both, except for UV-treated *Tenebrio molitor* powder, which has a limit of 1 lg CFU/g (10 cfu/g)(EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA Panel) et al., 2023).

Upon examining the coliform counts in dried pupae and comparing them to the *Enterobacteriaceae* counts shown in the insect-based novel food reports, satisfactory results were only observed for Farm 2, where no coliform contamination was detected. Farm 1 presented counts below 100 cfu/g (2 lg CFU/g), which were still considered acceptable in most insect-based novel food reports. However, both Farm 3 and Farm 4 exhibited at least one sample with values exceeding 2 lg CFU/g. When examining mesophilic bacteria (Total viable count) values of dried pupae, it was observed that all samples, except for one from Farm 3, had counts below 10^5 cfu/g. This limit was reported for mesophilic bacteria in all available reports. The study's findings align with previous research in the literature, which consistently shows that the implementation of a hot air-drying process effectively reduces the microbial counts of insects (Yan et al., 2023; Dandadzi et al., 2023). The results support the effectiveness of the drying process in reducing microorganism levels, highlighting its potential as a valuable method for improving the quality and safety of insect products.

Differences among farmers were not consistent along the rearing period.

Environmental microbiological data

The observed differences in environmental contamination among farmers may be partly attributed to the various supports and instruments used in silkworm rearing. The sampled surfaces, including jute bags, polymeric materials, paper, and metal fences exhibit significant differences across farmers. Each of these materials can affect the growth of microorganisms differently, depending on their ability to retain moisture and the surface area they provide for microbial colonisation. For instance, jute bags may have a higher level of transpiration, which could affect humidity levels, but they also offer a larger surface area for microorganisms to

grow. On the other hand, fences may limit microbial growth compared to other materials by minimising the available surface. It is important to note that these materials are used as supports to hold mulberry branches in a grid pattern during silkworm rearing.

Therefore, due to the diversity of supports and their distinct characteristics, the microbial environment may exhibit notable differences among farmers. It is important to take into account this variability when interpreting and comparing environmental contamination data. Understanding the influence of different rearing materials on microbial growth is crucial for optimising sericulture practices and ensuring a healthy environment for silkworms during their growth and development.

Correlation between rearing conditions and microbiological data

As expected, this study provides evidence that temperature and humidity have an impact on environmental microbial growth. Specifically, higher temperatures appear to promote the growth of coliforms, while elevated humidity levels seem to support the growth of moulds. Although no direct correlation was found between coliforms and moulds, the data suggest that high humidity levels may promote mould growth at the expense of other microorganisms, such as coliforms.

However, when examining microbial growth in larval samples, the clear impact of temperature and humidity observed in environmental samples was not evident. This inconsistency is likely due to the involvement of more intricate factors, such as the silkworms' diet and the microbial flora present in their digestive tracts. The relationships between the additional variables and the microbial composition and growth in the larval samples may be less straightforward compared to the environmental samples due to possible interactions.

Production performance indicators

As it regards the production performance indicators, the survival rate and quantity of fresh cocoons per batch observed in this study are consistent with historical data recorded by CREA-AA researchers over the last few years. The quantity of first-quality fresh cocoons, although highly variable among different breeders, also fell within the variability observed in previous years. Furthermore, the silkworm larvae in the current study did not exhibit alarming levels of specific diseases. Based on the farming performances observed in this study, it is likely that the observed levels of microbial population did not have any detrimental effects on the silkworms.

Overall considerations, limits and next steps

This study focuses on a limited number of microbial indicators that are useful for monitoring from a food safety perspective. However, it is likely that both pathogenic and non-pathogenic microbes make up the whole silkworm microbiota and that some of these microbes are symbionts that colonise the silkworm gut and participate in physiological processes.

As reported in the literature, lepidopteran gut microorganisms could be involved in a variety of physiological processes ranging from host nutrition and detoxification to immunity and protection by secreting antimicrobial peptides and outcompeting pathogens (Shao et al., 2024).

For future studies, it would be valuable to investigate the interaction between specific microorganisms and silkworms, as well as explore the potential effects of individual microorganisms on animal health. Such investigations can provide deeper insights into the complex relationship between microorganisms and silkworms, contributing to the optimisation of silkworm-rearing practices and enhancing overall farming outcomes.

As reported in the literature, the silkworm gut microbiota comprises four main phyla, Bacteroidetes, Actinobacteria, Firmicutes and Proteobacteria (Chen et al., 2018). Furthermore, the composition of the gut microbiota changes during larval growth, particularly between the first two and the last three instars (Chen et al., 2018). Shifts in the microbiota composition may also occur in response to environmental stress, such as the presence of heavy metals in mulberry leaves (Chen et al., 2023).

Microbial contamination could also originate from the rearing environment. The feeding process, which involves collecting mulberry leaves from the field, exposes silkworms to potential microbial contamination from the external environment. In addition, other insects may inadvertently enter the farm during this process and introduce further microbial challenges. In fact, the presence of other insects and small animals within the farm area cannot be ruled out. Farmers themselves could be a source of contamination, particularly concerning coliforms due to improper hygiene practices. As pointed out by Chen et al., the labour-intensive nature of sericulture could potentially expose the silkworm to a wide variety of non-indigenous microbes (Chen et al., 2018). Considering the various sources of contamination, farmers should implement strict hygiene practices and biosecurity measures to minimise the risk of microbial contamination. They should regularly monitor and take appropriate measures to ensure the health and safety of the silkworms and to maintain the overall quality of the rearing environment. By considering these factors, farmers can mitigate potential risks and optimise their silkworm-rearing practices. However, it is important to maintain an objective and balanced approach to the subject.

The current organisation of silkworm rearing, at least in the Veneto region, has both advantages and potential concerns that require careful consideration for future improvements, particularly in the perspective of using silkworm pupae as food. One positive aspect is that newly hatched larvae are provided by an independent research centre, and sanitary controls are conducted to ensure the initial healthiness of the silkworms. Furthermore, as the farmers' community remains relatively small, individual farmers can benefit from valuable assistance and professional training to enhance their rearing practices. This personalised support can lead to improved overall rearing efficiency and quality.

However, some critical points in the current organisation require attention. One concern is the practice of rearing silkworm larvae from the third instar, while the first two instars are managed by a nursery farm. This "weaning" phase involves transporting larvae between farms, which can expose them to contamination and stress, negatively impacting their health and development. Furthermore, conducting this phase in a single farm makes it vulnerable to disease outbreaks and contamination if improper practices are adopted, due to the high larval densities. To improve safety and efficiency, it is thus crucial to carefully select the most appropriate farm to rear the first instars while guaranteeing the application of preventive measures.

Conclusions

The collected data provide an important first step in investigating microbial contamination levels in silkworm farming and how they change throughout the rearing process. These data collected provide a foundation for future research, particularly in relation to the use of chrysalides as a new food source. Understanding the patterns of microbial contamination during the rearing process enables researchers to develop targeted safety measures to reduce contamination, starting from the first instars of silkworm larvae. A

significant finding from this study is that, although fresh pupae may occasionally exhibit high levels of microbial contamination, the drying process is remarkably effective in reducing microbial counts. This underscores the substantial potential of the drying process to enhance the safety and quality of silkworm pupae as a food product, at least for the microbial indicators investigated in this study. Furthermore, the study provides comprehensive insights into the specific procedures adopted by each farmer and their compliance to good practice guidelines in their respective farms. This information can help address sanitary concerns by discussing identified risk factors with farmers and during training courses. Future studies should focus on a more comprehensive and detailed characterisation of all microorganisms, with particular attention to pathogenic strains. A thorough characterisation of the microorganisms present in silkworm farms is essential to optimise rearing performances, ensure farm health, and enhance food safety. A deeper understanding of microbial dynamics can help the sericulture industry progress towards more efficient and safe practices, ultimately benefiting both farmers and consumers.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2024.101221.

Ethics approval

Not applicable.

Data and model availability statement

None of the data has been deposited in an official repository. Data are available on request from the first author.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

Author ORCIDs

Luca Tassoni: https://orcid.org/0000-0002-6529-5044.
Simone Belluco: https://orcid.org/0000-0002-3764-4040.
Filippo Marzoli: https://orcid.org/000-0001-8761-8206.
Barbara Contiero: https://orcid.org/0000-0002-6586-5035.
Alessio Saviane: https://orcid.org/0000-0003-4372-5688.
Silvia Cappellozza: https://orcid.org/0000-0002-7703-188X.
Antonella Dalle Zotte: https://orcid.org/0000-0001-9214-9504.

CRediT authorship contribution statement

L. Tassoni: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **S. Belluco:** Writing – review & editing, Formal analysis, Conceptualization. **F. Marzoli:** Writing – review & editing, Formal analysis, Data curation. **B. Contiero:** Methodology, Formal analysis. **S. Cremasco:** Writing – review & editing, Conceptualization. **A. Saviane:** Writing – review & editing, Writing – original draft. **S. Cappellozza:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **A. Dalle Zotte:** Writing – review & editing, Supervision, Conceptualization, Funding acquisition.

Declaration of interest

None.

Acknowledgements

We thank the committed farmers who actively participated in this study. Their contributions, including insights and samples from their silkworm farms, were essential to the scope and depth of our research.

Financial support statement

This work was co-funded by the CREA collaboration agreement "Metamorphoses" OB/FU 3.05.99.15.00 and by Padova University (DOR2022 - Dalle Zotte).

References

Cappellozza, S., 2010. Manuale di buona pratica agricola per la bachicoltura. CRA-API, Padua, Italy.
Chen, B., Du, K., Sun, C., Vimalanathan, A., Liang, X., Li, Y., Wang, B., Lu, X., Li, L., Shao, Y., 2018. Gut bacterial and fungal communities of the domesticated silkworm (*Bombyx mori*) and wild mulberry-feeding relatives. The ISME Journal 12, 2252–2262.
Chen, Y., Liu, G., Ali, M.R., Zhang, M., Zhou, G., Sun, Q., Li, M., Shirin, J., 2023. Regulation of gut bacteria in silkworm (*Bombyx mori*) after exposure to endogenous cadmium-polluted mulberry leaves. Ecotoxicology and Environmental Safety 256, 114853.
Commission Regulation (EU) 2017/893 of 24 May 2017 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council and Annexes X, XIV and XV to Commission Regulation (EU) No 142/2011 as regards the provisions on processed animal protein (Text with EEA relevance.). Retrieved on 24 May 2017, from http://data.europa.eu/eli/reg/2017/893/oj.
Commission Regulation (EU) 2021/1925 of 5 November 2021 amending certain Annexes to Regulation (EU) No 142/2011 as regards the requirements for placing on the market of certain insect products and the adaptation of a containment method (Text with EEA relevance). Retrieved on 5 November 2021, from: http://data.europa.eu/eli/reg/2021/1925/oj.
Dandadzi, M., Musundire, R., Muriithi, A., Ngadze, R.T., 2023. Effects of drying on the nutritional, sensory and microbiological quality of edible stinkbug (*Encosternum delgorguei*). Heliyon 9, e18642.
Delvendahl, N., Rumpold, B.A., Langen, N., 2022. Edible Insects as Food-Insect Welfare and Ethical Aspects from a Consumer Perspective. Insects 13, 121.
EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA), Turck D, Bohn T, Castenmiller J, De Henauw S, Hirsch-Ernst KI, Maciuk A, Mangelsdorf I, McArdle HJ, Naska A, Pelaez C, Pentieva K, Siani A, Thies F, Tsabouri S, Vinceti M, Cubadda F, Frenzel T, Heinonen M, Marchelli R, Neuhäuser-Berthold M, Poulsen M, Prieto Maradona M, Schlatter JR, van Loveren H, Ververis E and Knutsen HK 2021a. Safety of frozen and dried formulations from whole yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal. Retrieved on 23 October 2023, from https://data.europa.eu/doi/10.2903/j.efsa.2021.6778.
EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA), Turck D, Castenmiller J, De Henauw S, Hirsch-Ernst KI, Kearney J, Maciuk A, Mangelsdorf I, McArdle HJ, Naska A, Pelaez C, Pentieva K, Siani A, Thies F, Tsabouri S, Vinceti M, Cubadda F, Frenzel T, Heinonen M, Marchelli R, Neuhäuser-Berthold M, Poulsen M, Prieto Maradona M, Schlatter JR, van Loveren H, Ververis E and Knutsen HK 2021b. Safety of dried yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal. Retrieved on 23 October 2023, from https://data.europa.eu/doi/10.2903/j.efsa.2021.6343.
EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA Panel), Turck D, Bohn T, Castenmiller J, De Henauw S, Hirsch-Ernst KI, Maciuk A, Mangelsdorf I, McArdle HJ, Naska A, Pelaez C, Pentieva K, Siani A, Thies F, Tsabouri S, Vinceti M, Aguilera-Gómez M, Cubadda F, Frenzel T, Heinonen M, Marchelli R, Neuhäuser-Berthold M, Poulsen M, Maradona MP, Siskos A, Schlatter JR, van Loveren H, Zakidou P, Ververis E and Knutsen HK 2023. Safety of UV-treated powder of whole yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal. Retrieved on 23 October 2023, from https://data.europa.eu/doi/10.2903/j.efsa.2023.8009.
EFSA Scientific Committee 2015. Risk profile related to production and consumption of insects as food and feed. EFSA Journal 13, 4257.
Fasolato, L., Cardazzo, B., Carraro, L., Fontana, F., Novelli, E., Balzan, S., 2018. Edible processed insects from e-commerce: food safety with a focus on the *Bacillus cereus* group. Food Microbiology 76, 296–303.
Frentzel, H., Kelner-Burgos, Y., Fischer, J., Heise, J., Göhler, A., Wichmann-Schauer, H., 2022. Occurrence of selected bacterial pathogens in insect-based food

products and in-depth characterisation of detected *Bacillus cereus* group isolates. International Journal of Food Microbiology 379, 109860.
Han, R., Shin, J.T., Kim, J., Choi, Y.S., Kim, Y.W., 2017. An overview of the South Korean edible insect food industry: challenges and future pricing/promotion strategies: S. Korean edible insect food industry. Entomological Research 47, 141–151.
Huang, Y., Flint, S.H., Palmer, J.S., 2020. *Bacillus cereus* spores and toxins – The potential role of biofilms. Food Microbiology 90, 103493.
IPIFF Ensuring High Standards of Animal Welfare in Insect Production, 2019. Retrieved on 10 November 2023, from https://ipiff.org/wp-content/uploads/2019/02/Animal-Welfare-in-Insect-Production.pdf.
Klobučar, T., Fisher, D.N., 2023. When do we start caring about insect welfare? Neotropical Entomology 52, 5–10.
Lecocq, T., 2019. Insects: the disregarded domestication histories. In: Teletchea, F. (Ed.), Animal Domestication. IntechOpen, London, United Kingdom, pp. 35–67.
Li, G.-N., Xia, X.-J., Zhao, H.-H., Sendegeya, P., Zhu, Y., 2015. Identification and characterization of *Bacillus cereus* SW7-1 in *Bombyx mori* (Lepidoptera: Bombycidae). Journal of Insect Science 15, 136.
Marzoli, F., Antonelli, P., Saviane, A., Tassoni, L., Cappellozza, S., Belluco, S., 2022. *Bombyx mori* from a food safety perspective: a systematic review. Food Research International 160, 111679.
Mishra, N., Hazarika, N.C., Narain, K., Mahanta, J., 2003. Nutritive value of non-mulberry and mulberry silkworm pupae and consumption pattern in Assam, India. Nutrition Research 23, 1303–1311.
Mitsuhashi, J., 1997. Insects as traditional foods in Japan. Ecology of Food and Nutrition 36, 187–199.
Pali-Schöll, I., Binder, R., Moens, Y., Polesny, F., Monsó, S., 2019. Edible insects – defining knowledge gaps in biological and ethical considerations of entomophagy. Critical Reviews in Food Science and Nutrition 59, 2760–2771.
Pang-chuan, W., Da-chuang, C., 1988. Silkworm rearing. Food and Agriculture Organization of the United Nations, Rome, Italy.
Pasteur, L., 1870. Études sur la maladie des vers à soie : moyen pratique assuré de la combattre et d'en prévenir le retour. Gauthier-Villars, successeur de Mallet-Bachelier, Paris, France.
Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (Text with EEA relevance). Retrieved on 25 November 2015, from: http://data.europa.eu/eli/reg/2015/2283/oj.
Shao, Y., Mason, C.J., Felton, G.W., 2024. Toward an Integrated understanding of the lepidoptera microbiome. Annual Review of Entomology 69, 117–137.
Vasta, S., Figorilli, S., Ortenzi, L., Violino, S., Costa, C., Moscovini, L., Tocci, F., Pallottino, F., Assirelli, A., Saviane, A., Cappellozza, S., 2023. Automated prototype for *Bombyx mori* cocoon sorting attempts to improve silk quality and production efficiency through multi-step approach and machine learning algorithms. Sensors 23, 868.
Yan, X., Laurent, S., Hue, I., Cabon, S., Grua-Priol, J., Jury, V., Federighi, M., Boué, G., 2023. Quality of *Tenebrio molitor* powders: effects of four processes on microbiological quality and physicochemical factors. Foods 12, 572.
Yhoung-Aree, J., Puwastien, P., Attig, G.A., 1997. Edible insects in Thailand: an unconventional protein source? Ecology of Food and Nutrition 36, 133–149.