

Article

Study of Probiotic-Enriched Diet Effects on Yellow Mealworm Larvae Production at Laboratory and Pilot Scales

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Abstract

Global food security is an increasing challenge due to population growth and the limited availability of natural resources, driving the search for sustainable protein sources. In this context, edible insects such as yellow mealworms (*Tenebrio molitor*) have emerged as a promising alternative, while probiotics have been widely applied in animal production to enhance growth performance and nutritional quality. This study aimed to evaluate the effects of probiotic supplementation on the growth performance, biomass yield, and nutritional composition of yellow mealworm larvae at laboratory and pilot scales. Three probiotic strains—*Bacillus velezensis*, *Bacillus coagulans*, and *Pediococcus pentosaceus*—were tested at four different dosage levels, using wheat bran and brewer's spent grain as feed substrates. Larval growth was monitored weekly, and total harvested biomass, proximate composition (dry matter, protein, fat, and ash), amino acid profile, and mineral composition were determined using standardized analytical methods. At the laboratory scale, probiotic supplementation did not result in significant differences in mean larval weight or total biomass ($p > 0.05$). In contrast, at the pilot scale, significant improvements in larval growth and biomass were observed for specific probiotic treatments, with mean larval weights reaching approximately 140–150 mg and total harvest biomass increases of up to ~15% compared to the control ($p < 0.05$). Growth curves at both scales followed a sigmoidal pattern with a high correlation between laboratory and pilot experiments ($R^2 = 0.98$). Probiotic supplementation did not significantly affect crude protein content, but alterations in fat content, specific amino acid concentrations, and mineral composition were observed at the pilot scale, depending on strain and dosage. Overall, the results demonstrate that probiotic supplementation can enhance yellow mealworm production under pilot-scale conditions, while laboratory-scale trials may not fully capture these effects. These findings highlight the importance of scale when evaluating probiotic strategies and support the potential application of Bacilli-based probiotics to improve the efficiency and nutritional quality of industrial insect production systems.

Keywords: probiotics; yellow mealworm; scales; growth; proximate analyses



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1. Introduction

The growing global population, projected to reach nearly 10 billion by 2050, has posed significant challenges for sustainable food production and nutritional security [1]. Traditional agricultural systems are under immense pressure to meet rising demands while grappling with environmental constraints such as land degradation, water scarcity, and climate change. In this context, the identification of alternative, sustainable, and resource-efficient protein sources has become a priority for both researchers and policy-makers worldwide [2]. Among the proposed alternatives, edible insects have emerged as a promising solution, offering substantial nutritional, environmental, and economic advantages [2]. Insects are characterized by high feed conversion efficiency, low greenhouse gas emissions, and reduced land and water requirements when compared to conventional livestock systems [3]. Their potential as a mainstream food source has only recently gained attention in Western societies and industrialized food systems. Mealworms, in particular, have shown immense promise due to their high nutritional value, ease of cultivation, and low environmental impact [4]. Their protein content is comparable to that of traditional animal sources, while their environmental footprint is significantly lower, positioning them as a viable component of future food systems [5]. Among the various species of mealworms, the yellow mealworm (*Tenebrio molitor* Ln.) holds particular significance for human nutrition [6]. Yellow mealworm larvae are rich in high-quality protein, essential amino acids, lipids, vitamins, and minerals, making them a nutritionally balanced food source [3]. They are also notable for their relatively high content of unsaturated fatty acids, including omega-3 and omega-6 fatty acids, which are associated with cardiovascular and metabolic health benefits [7]. In addition, the presence of chitin contributes to dietary fiber, potentially supporting gut health, further enhancing the nutritional value of this insect species.

Probiotics play an essential role in nutrition and immune function in a wide range of organisms, including insects, and have been increasingly explored as dietary supplements to enhance growth performance and survival. According to the World Health Organization (WHO), probiotics are defined as ‘live microorganisms which, when administered in adequate amounts, confer a health benefit on the host’ [6,8]. Their routine application in agriculture and livestock production is primarily aimed at improving feed efficiency, promoting health, and reducing disease incidence. Previous studies evaluating probiotic supplementation in insect feeding trials have demonstrated beneficial effects such as enhanced nutrient metabolism, improved digestibility, increased biosynthesis of nutrients, and improved feed conversion ratios [9–13]. Research conducted on various species, including white shrimp (*Litopenaeus vannamei*), has shown that probiotics can improve intestinal morphology and digestive enzyme activity [14]. Similarly, studies involving *Ceratitis capitata* reported enhanced digestive functionality and successful gut colonization following probiotic supplementation [11]. In yellow mealworms (*Tenebrio molitor*), honeybees (*Apis mellifera*), and mud crabs (*Scylla paramamosain*), probiotic administration has been associated with improved immune responses and resistance to pathogens [8,13,15–17]. Despite these promising findings, there is still limited understanding of how commercially available probiotics—particularly those widely used in monogastric animal nutrition—perform when applied to insect production systems.

Furthermore, most existing studies have been conducted at laboratory scale, which may not adequately reflect the complexity of larger production systems. The scalability of probiotic effects from controlled laboratory conditions to pilot or semi-industrial scales remains insufficiently explored, especially with regard to growth performance, biomass yield, and nutritional composition of insects. This gap limits the translation of experimental findings into practical applications for industrial insect farming.

Therefore, the aim of this study was to evaluate the effects of probiotic supplementation on the growth performance, biomass accumulation, and nutritional traits of yellow mealworm larvae at both laboratory and pilot scales. Three probiotic strains were investigated at four different concentration levels: *Bacillus velezensis* (Calsporin®); *Bacillus coagulans* (PRBT-001®); and an experimental strain, *Pediococcus pentosaceus* (KVLB 19-01). In addition to assessing larval growth and total biomass, this study systematically analyzed proximate composition (dry matter, protein, fat, and ash), mineral content, amino acid profile, and protein profile, with the objective of understanding both the biological effects of probiotics and their scalability in insect production systems.

2. Materials and Methods

2.1. Insect Colony Preparation for Laboratory and Pilot-Scale Experiments

Insect colonies for both laboratory- and pilot-scale experiments were obtained from Bioinsect ApS (Salbjerg Vej 26, Havdrup, Denmark). A beetle density of 0.1 g cm^{-2} , as recommended in previous studies [18–20], was used for egg collection at both experimental scales. The laboratory-scale experiment was conducted at the Section of Organismal Biology (SOBI), University of Copenhagen (Frederiksberg, Denmark), whereas the pilot-scale experiment was carried out at the commercial farm where the beetles were sourced. For the laboratory-scale experiment, eggs were collected from homogenized adult beetle colonies aged between 3 and 15 weeks. To obtain eggs, 65.7 g of homogenously mixed adult beetles were placed in plastic trays ($16.5 \times 10 \text{ cm}$) and provided with organic oat flakes (365 Øko® Havregryn, Copenhagen, Denmark) as dry feed and 80 g of mushrooms as wet feed. Small Petri dishes filled with potato starch flour and covered with mesh (average mesh size: $1.62 \times 0.78 \text{ mm}$) were placed inside the trays for egg collection.

Oviposition was allowed for four days, after which the Petri dishes containing eggs were removed. This procedure was repeated in four trays to ensure sufficient egg production. The average egg mass obtained per tray was $5.46 \pm 0.47 \text{ g}$. Eggs from all trays were pooled and randomly distributed into plastic rearing boxes ($n = 65$; $16.5 \times 10 \times 7 \text{ cm}$) with vented lids, with 0.273 g of eggs per box (approximately 481 eggs, based on an average egg weight of 0.568 mg). Rearing boxes were maintained in a controlled insect incubator (Darwin Chambers®, St. Louis, MO, USA) at 27°C and 65–75% relative humidity under complete darkness.

For the pilot-scale experiment, 142.5 g of adult beetles was placed in an egg-laying tray ($49.5 \times 29 \text{ cm}$) with a mesh bottom, which was positioned directly inside a larval rearing tray ($60 \times 40 \times 14.5 \text{ cm}$). The tray contained 200 g of wheat bran, serving as both oviposition substrate and larval feed throughout the trial. Mushrooms were provided ad libitum as wet feed. After four days of oviposition, adult beetles and the egg-laying tray were removed, and eggs were allowed to hatch and develop in the rearing trays. The rearing room was maintained at 27°C with relative humidity between 65 and 70%. Carbon dioxide (CO_2) concentration was monitored using the Consibio® software (<https://www.consibio.com/>) and maintained below 2000 ppm through ventilation, in accordance with occupational safety guidelines. This was maintained on this level since high indoor CO_2 above 3000. 3,500,117 ppm could be detrimental to human health when working for a long duration of exposure [21].

2.2. Probiotics Strains and Culture of *Pediococcus pentosaceus*

Three probiotic strains were selected: two commercially available spore-forming *Bacillus* strains and one experimental strain isolated from mealworm gut microbiota. The *Bacillus* strains were chosen due to their documented resilience and effectiveness in animal nutrition [22–26]. These included *Bacillus velezensis* (Calsporin®, Calpis Ltd., Tokyo, Japan)

and *Bacillus coagulans* (freeze-dried powder PRBT-001[®], Creative Enzymes, Shirley, NY, USA). The third strain, *Pediococcus pentosaceus* (KVLB 19-01), was isolated from yellow mealworm guts at the University of Copenhagen. The cultivation, extraction, and freeze-drying of *P. pentosaceus* were performed according to previously described procedures [8,16]. Briefly, 100 mg of freeze-dried bacterial culture were suspended in 1 L of MRS medium and incubated at 37 °C for 24 h. Bacterial cells were subsequently washed three times with demineralized water by centrifugation at 3000× *g* for 15 min, resuspended in phosphate-buffered saline (PBS), and freeze-dried. This procedure was repeated until a total of 52.86 g of freeze-dried bacterial biomass was obtained. The bacterial stock concentration was 3.105×10^{11} CFU g^{−1} and was stored at −20 °C until use. Serial dilutions were prepared as described by Dahal et al. [8].

2.3. Experimental Design

A randomized block design was employed for both laboratory- and pilot-scale experiments. The experimental setup included a control diet without probiotics and probiotic-enriched diets at four dosage levels. These levels were defined as recommended/study-based dose (middle), double the recommended dose (high), half the recommended dose (low), and a fixed test dose, selected to allow for comparison across strains with different recommended inclusion rates. Five replicate rearing boxes were assigned to each treatment and dose level, resulting in 65 experimental units per scale (45 units in the first run and 20 in the second run) due to space limitations. The experiment was conducted in two consecutive runs separated by three weeks, using the same beetle population to ensure consistency. Eggs were homogeneously distributed across treatments to minimize initial variability. Dosage levels are detailed in Table 1. The fixed standard test dose was chosen for all the strains since these probiotic strains have differences in the scale of supplementation dosage. Five replicates (rearing boxes) were allocated for each treatment group under each dose level, which would make a total of 65 (45 + 20) experimental units at each setup. The experiment was run twice, 3 weeks apart; 45 experimental units in the first trial, followed by the remaining 20 units later because of rearing box limitations. For both runs, the same beetle populations were used and homogeneously distributed among the experimental units.

Table 1. Dose levels for probiotic strains used in the feeding trial.

Probiotics Strain	Recommended/Study-Based Dose/Middle)	Double the Recommended/Study-Based Dose (High)	Half of the Recommended/Study-Based Dose (Low)	Fixed Test Dose (Test)
<i>Pediococcus pentosaceus</i> (CFU/g)	2.5×10^9	5.0×10^9	1.25×10^9	1.0×10^9
<i>Bacillus velezensis</i> (CFU/G)	3.0×10^5	6.0×10^5	1.6×10^5	1.0×10^9
<i>Bacillus coagulans</i> (CFU/g)	5.7×10^6	11.4×10^6	2.9×10^6	1.0×10^9

2.4. Probiotic Feeding Trial and Larval Growth Measurements

At the laboratory scale, larvae were fed wheat bran as dry feed and mushrooms as wet feed during early development, followed by brewer's spent grain (BSG) from week five onward. Probiotics were administered once at week five, mixed thoroughly with wheat bran and incorporated into the diet. Larval growth was monitored weekly from week five to week nine, with 100 larvae randomly selected and weighed per replicate. Total larval biomass was also recorded. Larvae were harvested at week nine, starved for 48 h, and stored at −80 °C prior to analysis. At the pilot scale, feeding followed a similar protocol; however, probiotic-enriched feed was mixed directly with existing substrate, as complete feed replacement was not feasible. Larval sampling was performed using a spatial

subsampling approach, dividing each rearing tray into sections to ensure representativeness. Total biomass was measured only at harvest, due to logistical constraints

2.5. Chemicals and Larvae Selection

Proximate composition (dry matter, protein, fat, ash), amino acid profile, mineral composition, and protein profiling were determined using standardized AOAC, LC-MS, ICP-OES/ICP-MS, and SDS-PAGE methods, as described in detail in Sections 2.5–2.9. Statistical analyses were conducted using JMP Pro 16, applying nonlinear growth models, mixed-effects models, multiple linear regression, and ANOVA, with significance set at $p < 0.05$.

2.6. Proximate Composition: Dry Matter, Ash, Protein and Fat Content

Dry matter and total ash were determined according to AOAC [27]. Protein contents in the larvae samples were determined using Dumas's method (Elementar Analysensysteme GmbH, Langenselbold, Germany), as described by [28], with a nitrogen conversion factor of 4.76. Fat contents in the wet insect samples were determined by an Oracle universal fat analyzer (CEM Corporation, Matthews, NC, USA).

2.7. SDS-Gel Electrophoresis

The protein profile of the larvae samples was studied with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to [29], with slight modifications. In total, 120 mg of dried powdered sample was added to 2 mL of 1% sodium dodecyl sulfate (SDS) and SDS-solubilizing buffer, and it was homogenized with Polytron (Polytron PT 1200, Kinematica, Bohemia, NY, USA) for less than a minute to avoid foam creation in the sample. The sample was heated for 2 min, followed by cooling for 30 min at room temperature. The sample was heated again and homogenized with the addition of a Polytron. A 1 mL sample was then transferred to an Eppendorf tube and centrifuged in an Ole Dich centrifuge machine at $20,000 \times g$ rpm for 15 min. Afterwards, ca. 800 μL of supernatant (extract) was transferred into a new Eppendorf tube. The extract was subsequently diluted with $2 \times$ Laemmli sample buffer, in which 30 μL of it was mixed with a buffer solution ranging from 58 to 75 μL , depending on the requirement obtained after calculation, which depended on the protein % present in the insect samples. Afterwards, a required quantity of distilled water (Dw) ranging from 28 to 46 μL was added to the extract to achieve the required dilution. Finally, the solution was centrifuged at $30,000 \times g$ for 5 min at 20°C . After centrifuging, 10 μL of the solution was loaded into a well. Electrophoresis was performed on a 12% TGX Stain-Free polyacrylamide gel (Bio-Rad, Mississauga, ON, Canada) with a running buffer of $1 \times$ tris-glycine SDS solution (1.5 mm thick slab gels) (Bio-Rad, Mississauga, ON, Canada) at 150 V, 90 mA at 22°C for 1 h until the migration was complete. After completion of the program, the gels were taken out and washed with Dw by gently shaking them in a water bath. Before adding coloring agent to the gels, they were fixed with 40% methanol, 10% acetic acid and 50% water for 1 h. Afterwards, the gels were washed and ready for coloring with 50–100 mL of Bio-Safe Coomassie (Bio-Rad, Mississauga, ON, Canada). The protein-staining procedure was carried out for 1 h, followed by washing the stained gel with water for more than an hour until imaging. Molecular weight markers (Precision Plus Protein™ 161–0363; All Blue Unstained Protein Standards, Bio-Rad, Mississauga, ON, Canada) were used to estimate the molecular weight. Images of the gels were taken to analyze the protein band formation.

2.8. Amino Acid Composition

The total amino acid profile of the insect samples was determined with the LC-MS method, as described by [28]. Specifically, amino acid profiles were determined following

acid hydrolysis (6 M HCl) at 110 °C for 18 h using a sample concentration of 10 mg/mL. After cooling to room temperature (25 °C), hydrolysates were analyzed either undiluted or after a 3-fold (1 + 2) dilution with 6 M HCl to enable quantification of both high- and low-abundance amino acids. As tryptophan was degraded during acid hydrolysis, this amino acid was not included in the results. Subsequently, 100 µL of each sample was diluted with 1.5 mL of 1 M NaCO₃, filtered through a 0.2 µm syringe filter (Q-max PTFE, Ø13 mm, Frisette ApS, Knebel, Denmark), and derivatized using the EZ:Faast™ Amino Acid Analysis Kit (Phenomenex®, Torrance, CA, USA). Finally, 50 µL aliquots were analyzed by LC-(APCI)-MS (Agilent 1100, Agilent Technologies, Santa Clara, CA, USA).

2.9. Mineral Composition

Total mineral composition was determined according to Queiroz et al. [28], with modifications. Both macro- and micro-minerals were determined by inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma–optical emission spectrometry (ICP-OES), respectively, based on the principles of European standard methods EN 13805:2014, EN 15763:2009 and EN 16943:2017 [28]. Microwave-assisted digestion (Multiwave 7000, Anton Paar, Graz, Austria) was performed with 4 mL of HNO₃ (65% *w/w*; SPS Science, Villebon sur Yvette, France). The sample digests were diluted with ultrapure water (milli-Q) before analysis. Quantification was performed using yttrium as the internal standard. Macro-elements, such as Ca (calcium), Cu, Fe (iron), K (potassium), Mg (manganese), Mn, Na (sodium), P (phosphorus), S (sulfur) and Zn, were determined by ICP-OES (Agilent 5800 ICP-OES, Santa Clara, CA, USA). Likewise, micro-elements, such as As (arsenic), Cd (cadmium), Cu (copper), Hg (mercury), Mn (manganese), Pb (lead), Se (selenium) and Zn (zinc), were determined by ICP-MS (ICAPQ ICP-MS, Thermo Fischer Scientific, Bremen, Germany). Likewise, all standards were prepared from certified stock solutions (SPS Science) in 5% HNO₃. Accuracy and precision of both methods were evaluated by analysis with a CRM. Quality assurance of the analytical results was done by analysis of the certified reference material (DORM-4 fish protein, NRCC, Canada).

2.10. Statistical Analysis

Statistical analysis was performed using JMP Pro 16 (JMP Statistical Discovery LLC, 2024, Cary, NC, USA). The following statistical models were used for data analysis.

2.10.1. Larval Growth Curve

A nonlinear growth curve was used to analyze the growth pattern of the larvae in the experiment. Studies of holometabolous insects have shown their growth to be well explained by a sigmoidal curve [30]. Weight data that were taken at each week point was plotted against time, and the curve was plotted. The logistic growth model used in the analysis is as follows:

$$Y = \frac{c}{1 + e^{-a(x-b)}} \quad (1)$$

where ‘Y’ denotes the output variable, i.e., weight, and ‘x’ represents time in weeks. Similarly, small letters, a, b, and c, in the above equation denote growth rate (mg/week), inflection point, and asymptote, respectively. The inflection point is the point in the curve where the curve changes concavity (x₀,y₀). An asymptote that represents the point of stabilization or transition phase in larval growth is presented in the sigmoid curve (y = max), and Y_i is the measured weight (larval weight) in mg of the ith larva.

2.10.2. Linear Mixed Model

All response variables (except total harvest weight) were analyzed using a linear mixed model using Restricted Maximum Likelihood (REML) estimation. The statistical model used for data analysis [31,32] was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + f_k + \varepsilon_{ijk} \quad (2)$$

where Y_{ijk} represents the observed response variable for probiotic type i , dose level j , and replicate k ; μ is the overall mean; α_i is the fixed effect of probiotics; β_j is the fixed effect of dose level; $(\alpha\beta)_{ij}$ represents the probiotic $\times$ dose interaction, f_k is a random effect of replicates, and ε_{ijk} is the random error term. Two-way ANOVA was used to compare variances between and within the treatment groups. Afterwards, each pair-wise comparison was adjusted with the Tukey's HSD with a confidence interval set at 95% and was regarded statistically significant for $p < 0.05$.

2.10.3. Factorial Linear Model

Total harvest Y_{ij} was analyzed using factorial linear model. The statistical model [32] was given as:

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ij} \quad (3)$$

where Y_{ij} represents the observed response variable (total harvest) for probiotic type i , dose level j ; μ is the overall mean; α_i is the fixed effect of probiotics; β_j is the fixed effect of dose level; $(\alpha\beta)_{ij}$ represents the probiotic $\times$ dose interaction, and ε_{ij} is the random error term. Two-way ANOVA was used to compare variances between and within the treatment groups. Afterwards, each pair-wise comparison was adjusted with the Tukey's HSD with a confidence interval set at 95% and was regarded statistically significant for $p < 0.05$.

3. Results and Discussion

3.1. Larval Growth and Weight Dynamics at Laboratory and Pilot Scales

Figure 1 shows larval growth experiments at both the laboratory (Figure 1A) and pilot scales (Figure 1B). Both growth curves plotted with the mean weight recorded at each week followed a sigmoidal curve ($R^2 = 0.99$). Young insect larvae grew slowly until they reached 6 weeks old, which was followed by a rapid increase in weight over the next two weeks. After reaching week 8, there seemed to be a transition in the growth phase, where larvae tended to grow steadily or lose weight as they came close to pupation. Finally, when larvae reached this stage, the transition to the pupal phase was observed with no increment in weight, which is a biological phenomenon associated with molting into pupae. The growth tendency depicted in this study shows the characteristics of the dynamics of weight change (growth) in holometabolic insect species [30,33]. Larvae attained the fastest growth after the 7th instar until the 10th instar, which corresponds to the 6th week onwards until they emerge from their pupae [33]. This mechanistic growth in insect species has been shown to have a near-exponential trajectory, and the increase in growth is associated with energy reserves per biomass in later instars [34].

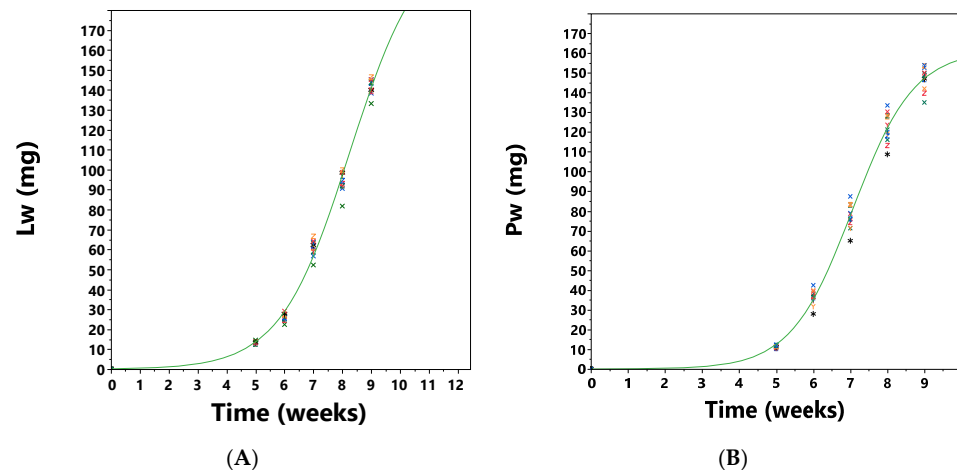


Figure 1. Growth curve of yellow mealworm larvae at laboratory scale (A) and pilot scale (B) from week 0 to 9 until harvesting. Y-axis represents mean weight per larva (Lw and Pw, mg) at laboratory and pilot scales, while X-axis represents time in weeks where growth in weight was recorded on a weekly basis. Data points at each week point are shown from week 5 to week 9, where mean weight per larva (mg) is plotted accordingly. Treatments and dose levels are designated with respective letters/legends and colors: Control (*), *Pediococcus pentosaceus* (X), *Bacillus velezensis* (Y), *Bacillus coagulans* (Z); green (recommended/study-based dose, middle), blue (double of recommended/study-based dose, high), red (half the recommended/study-based dose, low) and orange (test dose). The larval growth is fitted by the curve ($R^2 = 0.99$, RMSE = 3.88).

From the statistical analysis of the mean larval weight at week 9, the results did not show any effect of probiotic treatments on larval mean weight ($p > 0.05$) at the laboratory scale (Figure 2A). On the other hand, the study found significant differences ($p < 0.05$) in larval growth within and between the treatment groups (Figure 2B) in the pilot-scale trial. Compared to the control group, larvae from the BV and PP groups with high doses and the BC group with test doses were significantly heavier ($p < 0.05$). However, weight improvement was not observed for larvae in the BC (low) and PP (test and middle) groups. These differences could be explained by the larval density affecting the weight increment in insect larvae. Higher larval density (larval overcrowding) was noticed in some of the replicate boxes for those treatment groups. In those boxes, larvae were visibly smaller, implying the negative impact of high larval density on weight increment [35]. Another possible reason for not demonstrating probiotic benefits in nutrition could be the indoor climate in the rearing unit. Although the experimental setup was maintained as similarly as possible for both scenarios, climatic differences could not be avoided at the farm compared to the more controlled laboratory setup. At the farm, the temperature was recorded to go as high as 30 °C, and the relative humidity was up to 80%. Similarly, CO₂ levels could also reach above 2500 ppm, though ventilation was carried out daily. In addition, due to space limitations, complete climate control like in laboratories was not possible since routine farm production was also carried out in the vicinity. Studies carried out on insect growth have shown that several climatic factors, such as light, temperature, relative humidity, and ventilation, can influence insect larvae growth [36–40]. To support the tendency observed in this study at the pilot scale, the findings align with another study that found faster larval growth with higher humidity and temperature [38]. This tendency was more evident in the second batch at the pilot scale, in which larvae grew faster and attained a weight of over 100 mg after week 8 and were thus harvested at this week, whereas larvae were harvested at week 9 for the first batch. Similarly, a study done on the effect of higher CO₂ concentrations on larval development showed prolonged larval development. This prolongation could explain mass reserves in larvae, making them heavier, with a prolonged larval period to

pupation [40]. Figure 3 presents a correlation of the growth pattern between the pilot and laboratory scales. The line was fitted, showing high correlation ($r = 0.98$, $RMSE = 10.831$) between the two scales. The mean weight in the pilot scale is 1.14 times the mean weight of the laboratory scale. This means that the scale factor is 1.14, which shows that the mean weight at the pilot scale is only slightly higher than the mean weight at the laboratory scale. ($R^2 = 0.98$). Thus, this study shows that the translation of the probiotic feeding trial from a small setup to a larger scale could yield similar results in terms of larval growth. The above results on the nutritional advantages of probiotic supplementation at a pilot-scale increment are a novel breakthrough in the application of *Bacilli* strains, which are of animal-feed grade. The results for weight improvement in the insects align with previous studies, where other strains belonging to these species have been supplemented in other insects. Recent studies have shown that supplementing *Bacillus velezensis* EEAM in black soldier fly (BSF) larvae enhances feed conversion rates, larval weight, and protein content [23]. Additionally, various *Bacillus velezensis* strains have demonstrated benefits in other vertebrate species, such as increased egg production and improved feed conversion ratio (FCR) in laying hens [24]. Similarly, this bacterium has been shown to enhance growth, weight gain, and immune responses against *Vibrio parahaemolyticus* [22], as well as improve feed utilization and intestinal health [26] (Yang et al., 2024). On the other hand, this study was able to show that larvae fed with high (5×10^9 CFU) and low (1.25×10^9 CFU) doses of *P. pentosaceus* supplementation led to improved growth and biomass gain, aligning with previous studies that reported similar effects [8,16]. The dosage used in these studies (1×10^9 – 5×10^9 CFU) was comparable to the levels tested here. However, there was no beneficial effect at a dose of 2.5×10^9 CFU compared to the control. Based on this result, it can be assumed that the difference in concentration of bacteria does not significantly affect larval growth. Apart from overcrowding (high larval density) [35], the fungal infestation that was observed cannot be ignored in explaining these poor results in weight improvement in some treatment groups, such as BC (low) and PP (middle and test). Black dead bodies were observed in those boxes where mold growth was seen on the surface of brewer's spent grain, and those living were visibly smaller. Importantly, this mold growth could have negatively influenced insect growth, which was a similar scenario for BC (low). This could be related to an antagonistic impact on insect fitness in the presence of fungus, which could have caused reduced feed intake, thereby impairing growth, which was evident in this study [41]. On the one hand, mold growth hindered larval growth, but on the other hand, surviving larvae in groups such as BC (test), PP (high and low) and BV (high) were heavier. In this case, the lower larval density with other growth-favoring factors could have played a role in larval weight increment [35]. Thus, upon reflection on the results of this study, it can be emphasized that larval weight growth is influenced by complex environmental factors apart from the feed stuff used.

Following the feeding trial, total harvest weight was analyzed (Figure 4A,B). At the laboratory scale, no significant differences in total harvest weight were observed ($p > 0.05$) (Figure 4A). However, at the pilot scale, significant differences emerged depending on dosage levels across treatment groups (Figure 4B). Within these groups, compared to the control group, the high-dose BC treatment resulted in the highest harvest weight ($p < 0.05$), followed by PP (middle), while for the BV group, there were no significant differences ($p > 0.05$) among dose levels or from the control group (Figure 4B). Unlike the controlled laboratory environment, growth and nutritional variations were evident in farm-scale feeding, whereas for laboratory trials, we could not see the biomass boost with probiotic supplementation. The presence of yeasts and microorganisms in the feed could have masked the phenotypic effects of the probiotic strains [42]. However, at the pilot level, probiotics were found to promote survival and total biomass. It can be assumed that the

probiotic strains, particularly BC (high and low), PP (middle and test) and BV (low), may have synergistically acted with existing microbes in BSG, leading to overall benefits [43]. Additionally, environmental factors such as climate conditions and elevated CO₂ levels at the pilot scale may have facilitated *P. pentosaceus* proliferation in the feed, enhancing its effects compared to laboratory conditions [44]. However, on the other hand, in treatment groups such as BC (middle and test), PP (low and high) and BV (high and middle), most replicate boxes were antagonistically affected by mold growth. This caused lower harvest output, so it can be assumed that the secondary metabolites of fungi such as *Aspergillus* sp. and competitive interaction could have implicated larval survival and, hence, increased mortality, thereby lowering the larval number and, hence, the total biomass [42,45]. This tendency was observed during the trial as black dead larva bodies after week 7 and unused wheat bran and BSG in the tray, which gave a low biomass output.

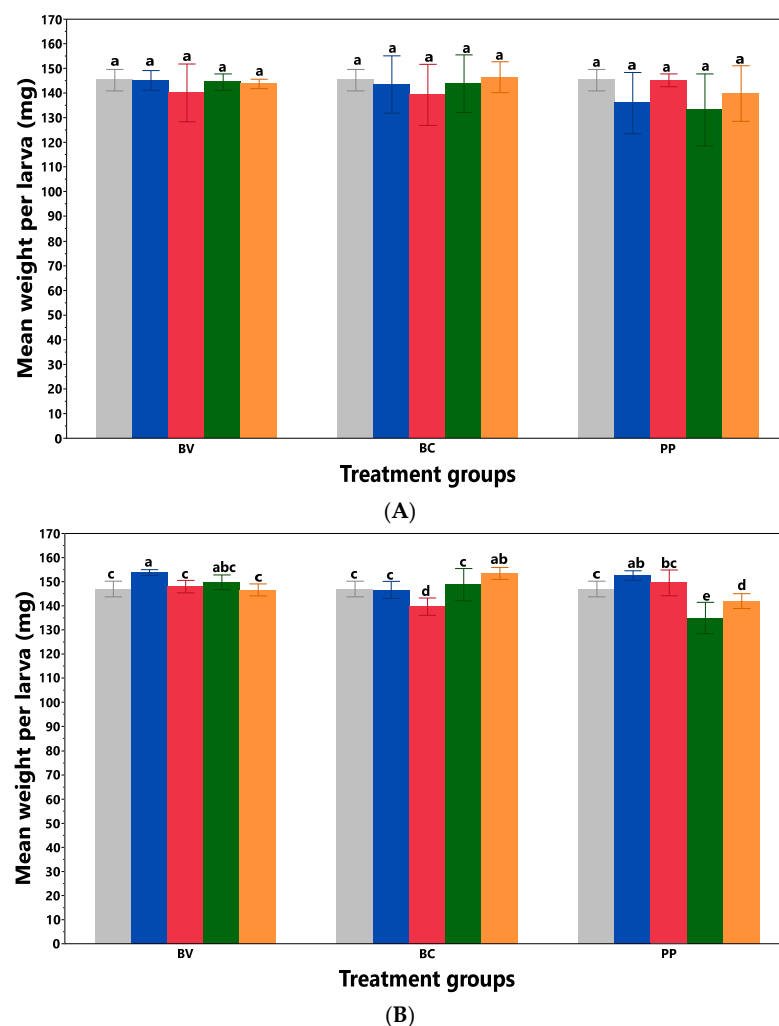


Figure 2. Mean weight graph at laboratory (A) and pilot scales (B). Mean weight of larvae in mg provided with different dose levels for each probiotic treatment group (*Bacillus velezensis* (BV), *Bacillus coagulans* (BC) and *Pediococcus pentosaceus* (PP)), compared within and between treatment groups with dose levels designated by colors; **Ctl**: control; **middle**: recommended dose for *Bacilli* strains and study-based dose for *Pediococcus* strains; **high**: double the recommended/study-based dose; **low**: half of the recommended/study-based dose; **test**: a chosen fixed dose for all the probiotic groups. The mean weight was determined by randomly selecting 100 and 500 larvae from each replicate box ($n = 5$) assigned for each dose level under each treatment group at laboratory and pilot scales. Small letters above the standard deviation bars denote statistically significant differences at $p < 0.05$.

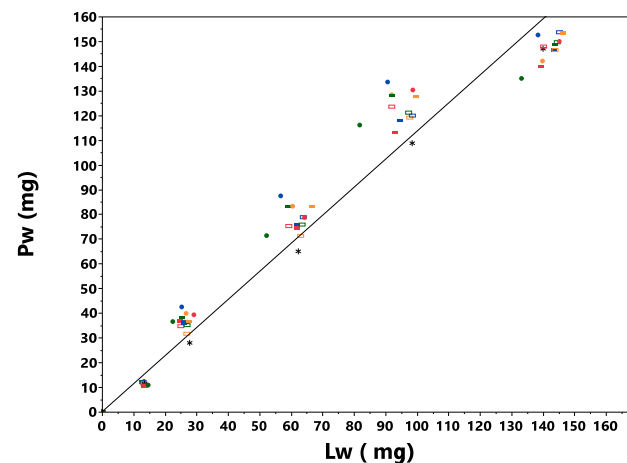


Figure 3. Larval growth correlation between industrial- versus laboratory-scale experiments. Pilot-scale larval mean weight (Pw, mg) plotted against laboratory-scale larval mean weight (Lw, mg). Treatments and dose levels are designated as follows: control (*), *Pediococcus pentosaceus* (O), *Bacillus velezensis* (■), *Bacillus coagulans* (□); green (recommended study-based dose, middle), blue (double of recommended/study-based dose, high), red (half the recommended/study-based dose, low), and orange (test dose). The growth model fits the line, as predicted by the model ($R^2 = 0.98$, correlation co-efficient (r) = 0.98, RMSE = 10.83). From the graph, mean weight at pilot scale is explained by the following equations: $y = 1.136x$, in which y and x denote the pilot-scale and laboratory-scale mean weight per larva, respectively.

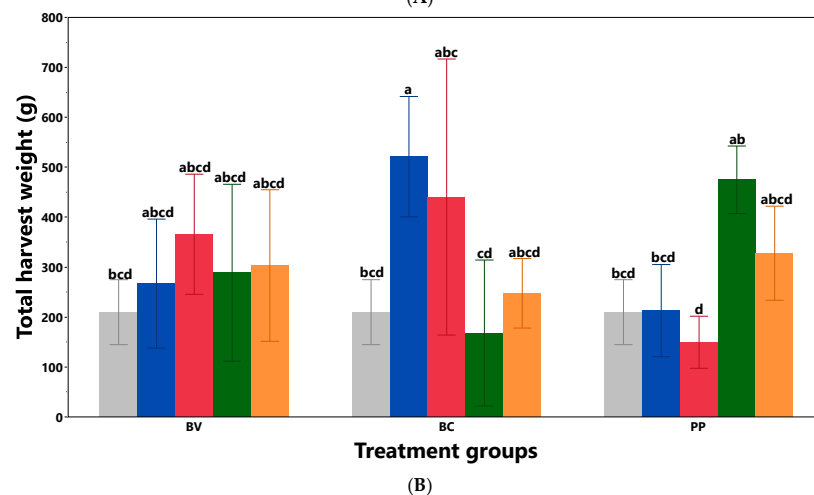
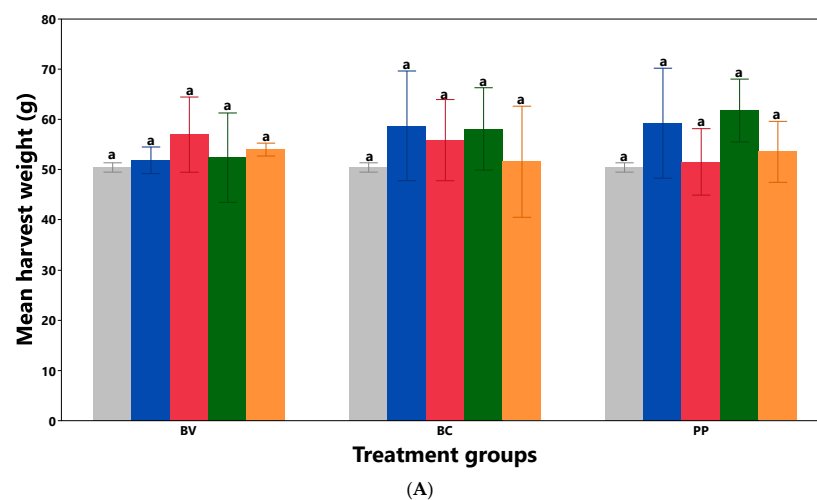


Figure 4. Mean harvest weight graph at laboratory (A) and pilot (B) scales. Mean total harvest weight (g) provided with different dose levels for each probiotic treatment group (*Bacillus velezensis* (BV),

Bacillus coagulans (BC) and *Pediococcus pentosaceus* (PP)), compared within and between treatment groups with dose levels designated by colors; **Ctl**: control; **middle**: recommended dose for *Bacilli* strains and study-based dose for *Pediococcus* strains; **high**: double the recommended/study-based dose; **low**: half of the recommended/study-based dose; **test**: a chosen fixed dose for all the probiotic groups. Small letters above the standard deviation bars denote statistically significant differences at $p < 0.05$.

3.2. Proximate Composition

The results of proximate composition analyses (dry matter, fat, protein, ash, and total amino acids) are presented in Supplementary Materials Table S1. At the laboratory scale, significant differences were observed only for dry matter and ash content, depending on treatment and dose ($p < 0.05$). No statistically significant differences were detected for crude protein, fat content, or total amino acid concentration among treatments ($p > 0.05$). At the pilot scale, significant differences were observed for dry matter and fat content ($p < 0.05$). *Pediococcus pentosaceus* at a low dose exhibited the highest fat content, while *Bacillus coagulans* at a high dose showed the lowest values. Crude protein and total amino acid concentrations did not differ significantly among treatments ($p > 0.05$), indicating that probiotic supplementation did not substantially alter protein deposition.

The results on dry matter and fat % found in this study align with previous findings by [46] on a wet weight basis. However, a higher crude protein % was reported compared to this study. Similar to the results obtained by [46], dry matter, protein and fat % were 35.2%, 17.4% and 13.4% respectively. However, the crude protein % found in this study is less than the values obtained in the study done by [6], in which dry matter (37.6–39.1%), fat (10.0–12.27%) and protein (14.8–17.4%) were reported. At both scales, differences were observed in dry matter and ash content, whereas protein and fat levels remained unaffected at the laboratory scale. However, at the pilot scale, fat content was influenced by probiotic supplementation. Interestingly, the fat content at this scale corresponded to the findings of [6] and was either comparable to or lower than that of the control groups. In the low-dose group supplemented with *Pediococcus pentosaceus*, fat content was reduced, suggesting potential hypolipidemic effects of probiotics, as previously reported by [6]. Although [6] reported an increment in protein % with probiotic supplements, the present study could not demonstrate such a pattern. This might be due to differences in the probiotic strains used in this study. Despite a similar experimental setup at both scales, differences could not be avoided in external climatic influences, and studies have shown that the elevated levels of CO₂ cause a high fat % in aphids [47]. Moreover, studies have reported that diet can greatly influence the fat level in insects such as yellow mealworm and the black soldier fly, but protein levels are regulated within narrow limits [48,49]. Another possible explanation could be the provision of brewer's spent grain (BSG) as wet feed, which naturally contains LAB, potentially leading to uniform protein levels across treatments [42]. Unlike that of Rizou et al. [6], this study did not assess nutritional composition on a dry-weight basis; this was instead carried out on a fresh basis, so the fat % could be affected by the moisture level in the present results. Here, differences in fat % in a small degree could be due to dry matter differences, and working on dry samples could have yielded different results on fat % than those of the present study. Apart from this, at the laboratory scale, although crude protein levels did not differ significantly among treatments, total amino acid levels were higher in the control group than in the probiotic-treated groups. This pattern was not observed at the pilot scale. One of the reasons for the reduction in total amino acid levels at the laboratory scale could be linked to the proteolytic activities of probiotic strains, which degrade chitin-binding proteins present in the exoskeleton, leading to lower amino acid concentrations in lactic acid bacteria (LAB)-treated larvae [50]. A recent study carried out on the black soldier fly (BSF) for chitin production showed that the bacterial protease enzyme

can also be used for the deproteinization of protein present as chito-protein matrices [51]. Similar mechanisms have been documented in shrimp, where *Bacillus mojavensis* facilitated the enzymatic deproteinization of insect exoskeletons through protease production [52]. A comparable process may have occurred during insect sample preparation, with serine protease activity in the hemolymph further contributing to amino acid degradation [53]. However, no such differences were observed in industrial-scale samples, indicating an absence of significant treatment effects on amino acid levels. Ash % content varied across treatment groups, ranging from 1.26 ± 0.0 to 1.11 ± 0.0 , lower than values reported in previous studies [54,55].

3.3. Mineral Composition

The mineral composition of various treatment groups was analyzed, and the results are summarized in Supplementary Materials Table S1. At the laboratory scale, PP (high) exhibited a significantly higher concentration of manganese (Mn) compared to the control group ($p < 0.05$). No statistical differences were observed among the other groups, except for PP (middle) and PP (low), which showed comparable levels to the control group. Copper (Cu) and zinc (Zn) levels were not significantly different from the control across all treatment groups, except for larvae from the PP (high) group. Similarly, there were no significant differences in calcium (Ca), potassium (K), sodium (Na), or sulfur (S) levels when compared to the control group. For iron (Fe), the PP (high) group recorded the highest levels, while the BV (middle) group had the lowest levels, both significantly different from the control ($p < 0.05$). However, other groups showed no significant differences in Fe levels compared to the control group. Regarding magnesium (Mg) and phosphorus (P) levels, the highest values were observed in the PP (high) group, and the lowest in the BV (middle) group. Despite this, neither group exhibited statistically significant differences from the control ($p > 0.05$; Supplementary Materials Table S1). Meanwhile, at the pilot scale, BC (test) and BC (high) showed the highest and lowest Mn levels, respectively, but they were not significantly different from the control ($p > 0.05$). A similar trend was also observed with Cu levels, where no treatment groups at different dose levels displayed significant differences from the control ($p > 0.05$). For Zn, Ca, and Fe levels, no statistically significant differences were observed across treatment groups, except for BC (high), which differed significantly from the control group ($p < 0.05$; Supplementary Materials Table S1). Potassium (K) levels showed no significant variation within or between treatment groups, except for BV (high), PP (middle), and BV (test), which were statistically different ($p < 0.05$) from the BC (high) group. For Mg, only PP (middle) and PP (high) exhibited statistically significant differences ($p < 0.05$) between themselves; however, these differences were not significant when compared to the control or other treatment groups (Supplementary Materials Table S1). Sodium (Na) and phosphorus (P) levels were highest in the BV (high) group and lowest in the BC (high) group, with a significant difference between these two groups ($p < 0.05$). However, no differences were observed when comparing these groups to the control or other treatments. Sulfur (S) levels across treatment groups did not significantly differ from the control, except for BC (high), which exhibited a significant difference ($p < 0.05$; Supplementary Materials Table S1).

In this study, mineral analysis revealed differences in both macro- and micro-mineral levels; however, the control group exhibited mineral levels nearly identical to those of the probiotic-treated groups. The mineral analysis was carried out on a dry basis, similar to other studies on mineral analysis in edible insects, in which experiments have been carried out on a dry matter basis [56]. This study showed differences in mineral contents, but this could vary per study since this can be influenced by food sources and breeding environments; these differences cannot be explained by differences in microbes in the

diet [56]. Research has shown that the nutrient composition of mealworms is influenced by diet [6,49]. Additionally, the ash content of wheat bran was reported to be relatively low (2.36 ± 0.96) by Rizou et al. [6], whereas studies on brewer's spent grain indicated higher values, ranging from 3.30 ± 0.04 to 4.29 ± 0.01 [57]. Furthermore, probiotics have been shown to play a role in mineral metabolism and interactions within the host [58], and differences in the level of these macro- and micro-minerals in this study could also have been influenced by probiotics [58,59]. Although no significant changes were observed in other nutritional parameters, dry matter and ash levels were affected at the laboratory scale, possibly due to probiotic-induced mineral regulation mechanisms, as observed in vertebrate studies [60]. Another possible explanation for mineral composition differences is bioaccumulation, as previous research on black soldier fly larvae has demonstrated their ability to accumulate metals from feed sources [61]. Despite a recent study carried out on BSFs that found the bioconversion of inorganic selenium to an organic form when selenium (Se) was added to their diet, that study could not demonstrate the presence of selenium in insect bodies upon mineral analysis [62]. Furthermore, fortifying mealworm diets with selenium could provide an organic source of this essential metal for human and animal consumption [63]. Moreover, the accumulation of metals in beetles occurs in tissues such as the hepatopancreas, malpighian tubules, midgut, ovarioles, and mandibles, but this depends on factors such as age, sex, physiology and genetics [64]. Thus, it is assumed here, in this study, that probiotics could have modified metabolic pathways in larvae, leading to bioaccumulation in the tissues as well. Such mechanisms have been found in studies where both lactic acid bacteria and *Bacilli* strains are able to bioremediate metal ions through biosorption and bioaccumulation [65,66].

3.4. Amino Acid Analysis

The concentrations of essential and non-essential amino acids were analyzed, with the results presented in Supplementary Materials Table S1. At the laboratory scale, the control group exhibited the highest levels of phenylalanine (Phe), leucine (Leu), histidine (His), and lysine (Lys), which were significantly higher ($p < 0.05$) compared to all treatment groups. The treatment groups did not show statistically significant differences from one another. For isoleucine (Ile), the control group recorded the highest level; however, it was not statistically different ($p > 0.05$) from the other groups, except for BV (low), BC (high and low), and PP (high). No significant differences were observed in methionine (Met), threonine (Thr), or cysteine–cytosine (C-C) levels among the treatment groups, regardless of dose levels. Valine (Val) levels were significantly higher in the control group compared to all treatment groups, except for BC (test) and PP (test). Regarding non-essential amino acids, tyrosine (Tyr) levels in the control group were significantly higher ($p < 0.05$) than those in all treatment groups, except for PP (test), PP (low), and BC (low and high). Similar trends were observed for proline (Pro), apart from the test dose groups for BC and PP, which were not significantly different from the control. Alanine (Ala), glycine (Gly), arginine (Arg), glutamine (Glu), and aspartate (Asp) levels were significantly higher ($p < 0.05$) in the control group compared to all treatment groups, while no statistical differences were found among the treatment groups themselves ($p > 0.05$). Serine (Ser) levels were also significantly higher ($p < 0.05$) in the control group than in other treatment groups, except for BV (high) and BC (test). At the pilot scale, no significant differences ($p > 0.05$) were observed in the levels of either essential or non-essential amino acids among the control group and treatment groups, regardless of dose levels, except for arginine (Arg). Compared to the control, the BC (middle) group exhibited the highest arginine levels ($p < 0.05$), although these were not significantly different from the levels observed in the BV (middle, low, and test) groups. This study did not show improvement in total amino acid levels at either scale

of experiment, despite observing differences in amino acids per dose level for probiotic treatments. In addition to this, this study could not demonstrate an improvement in both essential and non-essential amino acids, which is in contrast to other probiotic studies done on vertebrate animals, such as chickens [67]. The anabolic activities of probiotics have been associated with exogenous proteases, amino acids and peptide transporter activities, as well as increased intestinal villi morphology and metabolism for nutrient uptake [68]. Despite weight improvement observed at the pilot scale, a difference could not be seen in amino acid levels, which could be linked to protein regulation based on developmental stages in insects [33]. Studies have shown a decrease in protein levels, as well as total amino acids, including both essential and non-essential amino acids, with an increase in instar levels [33,69]. Here, a nutritional study was done on late instars (13th) before pupation, which showed decreased protein synthesis activities since metabolic activities are downregulated in preparation for pupal molting [33]. Moreover, larvae in the probiotic groups gained weight faster than control larvae, which was evident with increased pupal emergence compared with the control group, which could be related to the decreased total amino acids in this study. Thus, the effects of probiotics on amino acid levels should be determined at different instars (larval stages), which could show actual changes in amino acid levels rather than at harvest, as was carried out in this study.

3.5. SDS-PAGE

Figure 5 shows the protein band distribution. All groups exhibited similarities in protein band formation. PP with middle (T21) and high doses (T22) and the BC group with a low dose (T43) showed light-colored protein bands. An intense band was found close to 70, 40, 25 and 30 kDa. Large, intense bands were formed at 10 and 15 kDa. Similarly, Figure 5B shows the protein distribution in SDS-PAGE, with columns representing treatment groups with different dose levels. No clear protein band was visible for the control group (T1), BV with middle (T31) and high (T32) doses, or the BC with middle dose (T41) group. The rest of the treatment groups with different doses showed similarities in protein band formation. A light band was found close to 70, 50, 40, 25 and 30 kDa. Large and intense protein bands formed between 10 and 15 kDa. The intense and large protein band ≤ 14 kDa could possibly originate from anti-freeze proteins, which ranged from 8.5 to 13 kDa, and hemolymph proteins [70]. A similar mechanism was observed for the samples of *Bacillus velezensis* (high dose). The bands, ranging from 14 to 32 kDa, are from *T. molitor* cuticular proteins. In addition, the band observed at 23 kDa could be attributed to chymotrypsin-like proteinase [71,72]. Similarly, three protein bands observed between 37 and 50 kDa could be linked to enzymes and other proteins, e.g., melanization-inhibiting protein (43 kDa), alpha-amylase (~50 kDa, a putative allergen), and muscle proteins such as actin-like and Troponin T (e.g., actin 30–50 kDa) [73,74]. Furthermore, three distinct protein bands were found between 50 and 75 kDa. Those proteins could be tubulin α – 1 chain (50.6 kDa), putative uncharacterized protein (Myosin tail of 60.1 kDa), and calcium transporting ATPase (72.9 kDa); furthermore, a protein band at 75 kDa was observed that could be attributed to tropomyosin 1 [73].

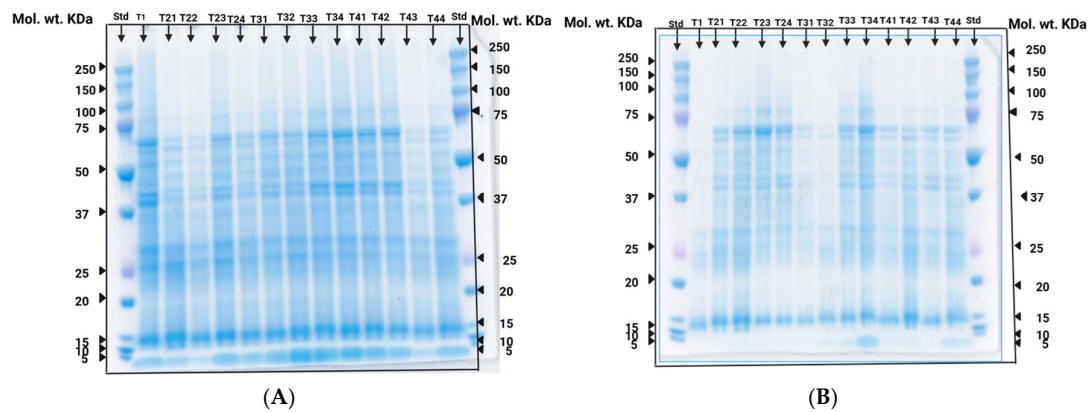


Figure 5. SDS-PAGE for insect samples at laboratory (A) and pilot (B) scales. Each column with bands denotes treatment groups with four dose levels. Here, Std represents the standard (marker) used to compare the molecular weight for each band. Here, treatment T1 is the control group, while the first digit followed by treatment (T) for other columns represents test probiotic strains: *Pediococcus pentosaceus* (2), *Bacillus velezensis* (3), *Bacillus coagulans* (4). The second digit represents the dose levels. Recommended/study-based/middle (1), double the recommended dose/high (2), half of the recommended dose/low (3), and fixed test dose/test. The protein band for the std (marker) column is provided by the manufacturer.

4. Conclusions

This study evaluated the effects of probiotic supplementation on the growth performance, biomass yield, and nutritional composition of yellow mealworm (*Tenebrio molitor*) larvae at laboratory and pilot scales. The results demonstrated that probiotic supplementation did not significantly influence larval growth or total biomass under laboratory-scale conditions, indicating that highly controlled environments may limit the observable benefits of probiotic application. In contrast, at the pilot scale, specific probiotic treatments resulted in significant improvements in larval growth and biomass yield, with mean larval weights reaching approximately 140–150 mg and total harvest biomass increases of up to ~15% compared to the control ($p < 0.05$). These findings suggest that probiotic effects become more evident under semi-industrial rearing conditions, where environmental variability and microbial interactions are more representative of real production systems.

Growth curves at both experimental scales followed a sigmoidal pattern and showed a strong correlation ($R^2 = 0.98$), indicating that laboratory-scale experiments can reliably predict general growth trends, although they may underestimate absolute performance metrics observed at larger scales. This highlights the importance of validating laboratory findings under pilot or industrial conditions before practical application. Regarding nutritional composition, probiotic supplementation did not significantly alter crude protein content or total amino acid concentration at either scale, suggesting that the primary impact of probiotics was related to growth efficiency rather than protein deposition. However, strain- and dose-dependent variations in fat content and mineral composition were observed at the pilot scale, indicating potential metabolic or microbial interactions that warrant further investigation. Overall, the findings support the potential use of Bacilli-based probiotics as functional feed additives in yellow mealworm production systems, particularly at pilot or industrial scales. Future studies should focus on controlling environmental variables, evaluating long-term production cycles, and elucidating the mechanistic interactions between probiotics, gut microbiota, and insect metabolism in order to optimize probiotic strategies for sustainable insect farming.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/app16041800/s1>, Table S1. Laboratory and pilot scale results for *Pediococcus pentosaceus*, *Bacillus velezensis* and *Bacillus coagulans*.

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