Spermidine Enhances Nutritional Indices of *Bombyx mori* (Lepidoptera: Bombycidae) Larvae[1]

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Abstract The demand for silk increases as its economic value rises. Silk production by the silkworm, *Bombyx mori* L., is linked to the quality of the silkworm diet, leading to efforts to nutritionally enrich the mulberry (*Morus alba* L.) diet of silkworm larvae. Previous studies have established that spermidine, a polyamine, enhances larval growth in *B. mori*, subsequently increasing silk production. However, its role in improving the nutritional quality of the silkworm diet is not known. In this study, we evaluated the effects of spermidine-treated diet on the nutritional indices, polyamine levels, and antioxidant potential in fifth-instar larvae. We also assessed the effect of consumption of the spermidine-treated diet on the larval gut microbiome, which impacts digestion and assimilation of nutrients. Larvae consuming the spermidine-treated diet showed a significant increase in the efficiency of conversion of ingested food and digested food, intracellular polyamine levels (especially the conjugated and free fraction), antioxidant potential and cell viability, and both diversity and number of bacterial communities. These findings suggest that feeding mulberry leaves fortified with spermidine enhances nutritional efficiency in the *B. mori* larvae and may represent a method of increasing silk production by *B. mori*.

Key Words *Bombyx mori*, gut, nutrition, polyamine, spermidine

The domesticated silkworm, *Bombyx mori* L., is an economically important insect that feeds on mulberry leaves and produces silk. The food consumed by larvae determines the quality and quantity of silk produced (Etebari and Matindoost 2005, Gobena and Bhaskar 2015). The dietary requirements of the silkworm are met by digestion and assimilation of nutrients present in the mulberry leaves. The presence of certain gut bacterial flora further influences larval growth, absorption, and nutrient utilization (Nagaraju 2002). The final stage of larval development (e.g., fifth-instar larvae) before cocoon formation is especially important because it is during this stage that the number of expressed proteins is at its highest (Broderick and Lemaitre 2012, Lemoine et al. 2020). At this stage, the silkworm larvae consume an estimated 85% of the total diet consumed during larval development. This period of development coincides with rapid tissue growth and peak silk production. Nutritional supplementation at this stage alone enhances silk production (Mastan et al. 2017) and is thus promising from an economic perspective. All living organisms have natural polyamines like cadaverine, putrescine, spermidine, and spermine

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(Bachrach 1973, Bardócz et al. 1995, Pegg 2016). Polyamines participate in major cytologic processes such as replication, transcription, translation, membrane stability, cell proliferation, differentiation, apoptosis, and tumorigenesis (Tsukada et al. 2005, Mondal et al. 2007). Our laboratory has previously shown that spermidine treatment improves the quality (Yerra et al. 2017) and quantity of silk (Lattala et al. 2014, Yerra and Mamillapalli 2016), increases metabolic activity and decreases oxidative stress in larval silk glands (Yerra et al. 2016), and increases egg production in *B. mori* (Mysarla et al. 2016a, 2016b). In this study, we hypothesized that spermidine supplementation at the fifth-instar larval stage would be a beneficial and nutritionally efficient addition to the traditional simple mulberry diet. To test this, we assessed the effects of spermidine on larval growth, nutritional efficiency, polyamine quality, antioxidant potential, and microbiome contribution.

Materials and Methods

Newly ecdysed fifth-instar larvae were selected from a bivoltine CSR2 × CSR4 colony of *B. mori* maintained by Andhra Pradesh State Sericulture Research and Development Institute (Hindupur). We continued to maintain the larvae at 26 ± 2°C and a relative humidity of 65–85% following the methods of Lattala et al. (2014). Selected larvae were surface cleaned and grouped as day 0. Larvae were allowed to feed the next day of ecdysis and considered as day 1. Mulberry leaves were fortified with 50 μM spermidine by swabbing the leaf surfaces (RM 5438; Himedia Laboratories, Mumbai, India) and were fed to the treated group, whereas a mulberry diet without spermidine was fed to the control larvae for 7 d. The present study was conducted with 50 μM polyamines, as the standardization of all the polyamines over a range of concentration was performed in an earlier study by Lattala et al. (2014).

After 7 d of feeding, control and spermidine-treated larvae were surface sterilized with 75% ethanol and rinsed with sterile distilled water. Larvae were dissected in sterile insect Ringer solution (0.85% NaCl), and the entire gut (foregut, midgut, and hindgut) was removed aseptically. The gut contents were thoroughly rinsed, and the gut tissues were homogenized and used for further analyses.

Nutritional indices. The gravimetric technique was used to determine weight gain, food consumption, digestibility, and ingestibility of food for both control and spermidine-fed larvae. A total of 500 larvae was used for this analysis as the experiment was performed five times with five different generations of larvae. For each generation group and experimental set, 200 individuals were sampled in both control and spermidine-treated groups (100 larvae each in control and spermidine-treated groups). For ease of handling and accuracy of measurement, the larvae in both groups were maintained as separate pools of 10 larvae each in 10 separate trays. The larval weight, fresh leaf weight, food, and frass remaining at the end of each day were recorded until the larvae reached the prepupal stage (day 6). These measurements were performed five times with five different generations of larvae. The dry weight of fresh leaves (before feeding), larvae (before feeding), remaining dry leaves, and frass were measured after drying at 60°C for 24 h (Waldbauer 1968). Consumption index (CI), approximate digestibility (AD), efficiency of conversion of ingested food (ECI), and efficiency of conversion of digested food (ECD) were calculated with the following equations: $CI = E/A$, $AD = (E - F)/E$, $ECI =$

P/E and ECD = P/(E − F), where A = mean dry weight of insect over unit time, E = dry weight of food consumed, F= dry weight of frass produced, and P = insect dry weight gain.

Gut polyamine measurement. Free, conjugated, and bound polyamines were analyzed from the gut samples of control and spermidine-treated larvae. A pool of 40 larvae each in the control and treated groups was maintained, and 10 larvae were processed at days 1, 3, 5, and 7 for each experimental set. A total of 400 larvae were used for this analysis, which was conducted in five replicative sets with five different generations. The entire gut was homogenized with 10% perchloric acid and centrifuged at 15,000 × *g* for 15 min at 4°C. To prepare conjugated, bound, and free fractions of polyamine, the supernatant and pellet were processed and loaded onto thin-layer chromatography plates coated with silica gel as per protocols of Bharti and Rajam (1995). The dansylated polyamine bands were observed under ultraviolet (UV) light and marked. Individual polyamine bands were scrapped off the plate, eluted in ethyl acetate, and quantified with a spectrofluorometer with an excitation at 350 nm and emission at 495 nm.

Antioxidant assays. Antioxidant assays were performed to compare the free radical scavenging potential of spermidine-treated larvae and control larvae. A total of 300 larvae were used for this analysis, which was conducted in five experimental sets from five different generations of larvae. A total of 60 larvae in each group was maintained, and 5 larvae from each group were sampled for the assays on days 1, 3, 5, and 7. The total antioxidant ability was measured using the ferric reducing antioxidant power (FRAP) assay (Wong 2006), the 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay (Zhao 2010), and superoxide dismutase (SOD) assay (Beauchamp and Fridovich 1971).

Cell viability assay. Cell viability with gut samples of larvae fed on spermidine was determined using the assay based on the reduction of the dye 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by cellular enzymes produced by viable cells. A total of 200 larvae were used for this analysis. Control and spermidine-treated groups with 20 larvae in each group were used in this assay, and 5 larvae were processed on days 1, 3, 5, and 7. This assay was performed five times with five generations of larvae. The gut samples were processed as per the established protocol (Nath et al. 2005). The optical density was measured at 570 nm using a spectrophotometer. MTT solution without the gut sample was used as a blank.

Gut bacteriota. Guts were dissected from control and treated larvae, and the contents of each were emptied and homogenized in 1× phosphate buffer solution. The homogenates were serially diluted and drop-plated onto nutrient agar plates, which were incubated at 37°C for 24–48 h to evaluate maximum cultivable bacteria, and identification of total noncultivable bacteriota was conducted using Illumina platform (Rajan et al. 2020). The 10^{-7} dilutions yielded 200–400 distinct colonies per plate and were used to enumerate the number of bacterial colony-forming units (CFUs) per milliliter in the original gut homogenate samples. A total of 300 larvae were dissected with 150 from the control group and 150 from the spermidine treatment. Five groups of 30 larvae comprised each treatment group as the procedure was repeated five times from five different generations.

Thirty individual colonies were selected and isolated from each group for cultural, morphologic, and biochemical characterization. Characterization of all the isolated

bacterial colonies from control and spermidine-fed groups was performed using standard techniques. Cultural characteristics such as size, shape, and pigmentation were studied after incubation at 30°C for 24–48 h. Gram staining was performed to study the morphology of the collected isolates. Biochemical characterization was conducted with indole acetic acid, methyl red, Voges–Proskauer, citrate utilization, urease test, catalase, oxidase, H_2S production, gas production from glucose, and nitrate reduction tests. The identification of the colonies was based on Bergey's Manual of Systemic Bacteriology (Krieg et al. 2010, Sneath et al. 1986).

Statistical analysis. Data from these tests were subjected to a Student's *t* test assuming unequal variances to determine any statistical significance ($P < 0.05$) between control and spermidine treatments.

Results

Larval and gut growth response. To test the effect of spermidine consumption on *B. mori* larval and gut growth, larval weights and gut weights (Fig. 1A, B) were measured on alternate days of development of the fifth instars. Larval and gut weights before the exposure to spermidine were recorded as day 0. Spermidine-treated larvae displayed a significant increase ($t = 2.16$; df =13; $P < 0.00002$) in body weight from day 1 to day 5 compared with the control larvae. Although gut weights of control larvae (from day 1 to day 7) remained relatively unchanged, gut weights of spermidine from day 1 to day 5 increased significantly ($t = 4.3$; df =2; $P = 0.03$) at day 7. Spermidine-fed larvae ceased increasing gut weight on day 7 (Fig. 2). Spermidine-fed larvae displayed enhanced growth and early pupation compared with the control larvae, suggesting that spermidine supplementation in the diet accelerates the development of larvae and reduces the time required for silk production.

Nutritional indices response. Because growth is dependent on the type and amount of diet consumed and the efficiency with which it is used, we measured and compared the nutritional indices of the control and spermidine-fed larvae. Nutritional indices of the spermidine-fed group showed a highly significant increase in ECI ($t = 3.18$; df = 3; $P = 0.003$) and ECD ($t = 3.18$; df = 3; $P = 0.004$) compared with the control group (Fig. 3A, B). The CI of the spermidine-fed group showed a relative decrease ($t = 2.35$; df = 3; $P = 0.002$) compared with the control group, whereas AD did not show any significant difference between control and spermidine-treated groups (Fig. 3C, D). These results indicate that spermidine-fed larvae exhibited improved and efficient utilization of the diet compared with control larvae. This may have allowed the larvae to grow more rapidly and reach the silk spinning stage sooner than those fed on mulberry diet alone.

Polyamine response. To test the effect of spermidine consumption on the different forms of intracellular polyamine levels, we quantified conjugated, free, and bound levels. Conjugated polyamine was the predominant form compared to free and bound polyamine in both control and spermidine-fed gut tissues. The levels of conjugated polyamine increased from day 1 to day 3 and then decreased. Conjugated polyamine levels in guts from spermidine-fed larvae were significantly higher ($t = 2.11$; df = 16; $P < 0.0001$) than levels in control guts on days 3, 5, and 7. Supplementation of spermidine significantly increased free polyamine ($t = 2.16$; df =

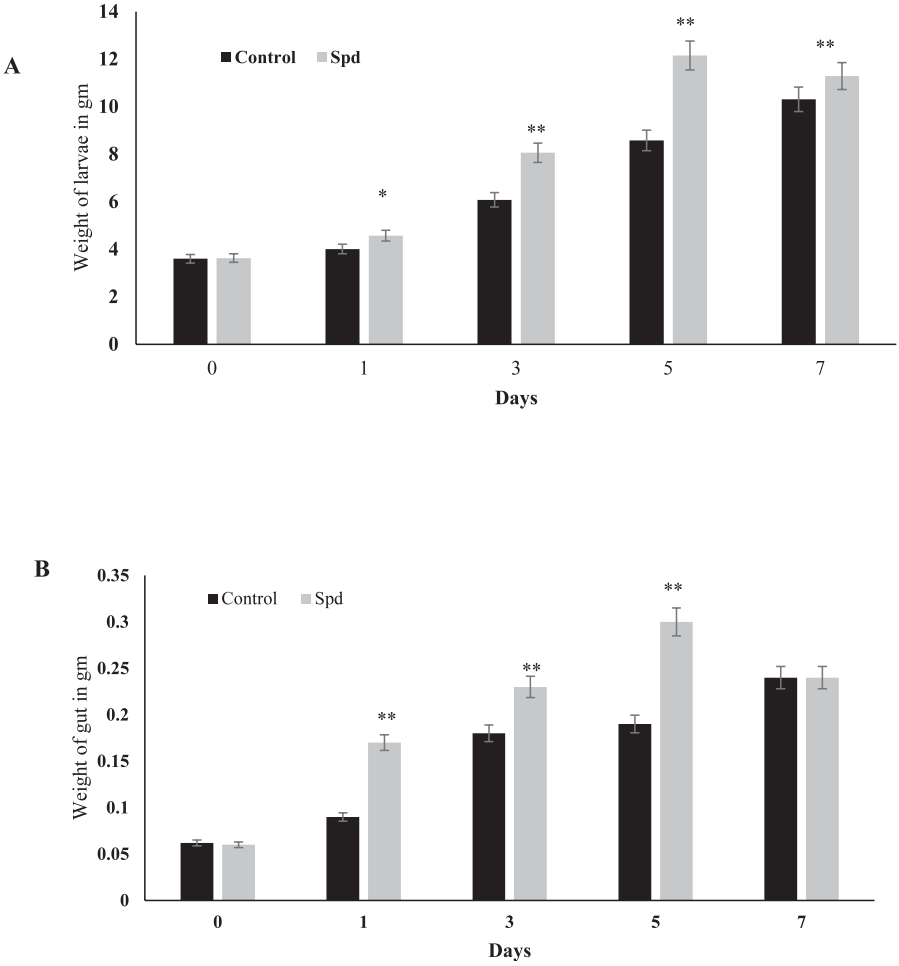

Fig. 1. Effect of spermidine (Spd) on growth of *B. mori* larvae. (A) Effect on larval weight during fifth-instar larval development. (B) Effect of on gut weight. Day 0 represents the weight before treatment after the fourth molt. *Significantly different ($P < 0.05$). **Highly significant ($P < 0.01$) in comparison with control larvae.

13; $P = 0.0001$) on all days of fifth-instar larval development compared with control. The total quantification of endogenous gut polyamine concentration (putrescine, spermidine, and spermine) confirmed that the spermidine supplementation augmented the intracellular polyamine levels (Fig. 4).

Oxidative stress response. Conjugated polyamines participate in scavenging of oxygen free radicals and protect DNA and phospholipids from oxidative damage (Minguzzi et al. 2019, Rhee et al. 2007, Rider et al. 2007). Thus, the effect of spermidine supplementation on the free radical scavenging ability was investigated

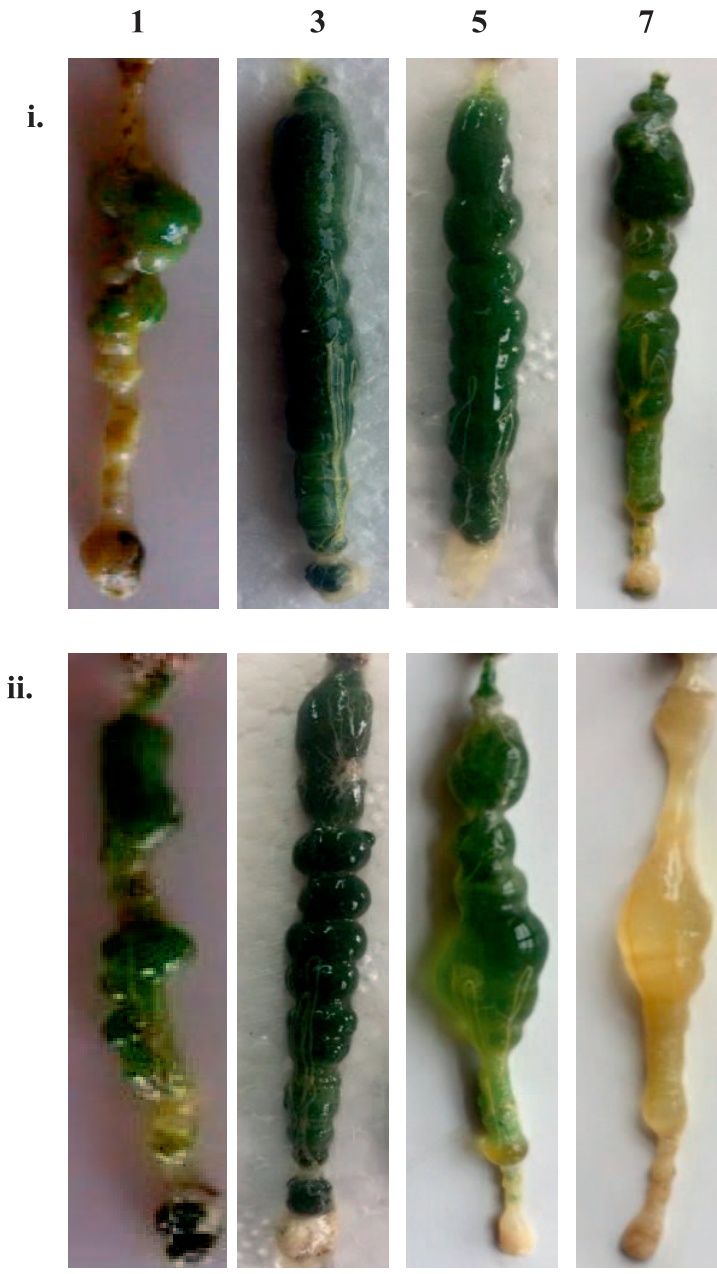

Fig. 2. Morphologic changes of guts of control (i) and spermidine-treated (ii) *B. mori* during fifth-instar development. (1, 3, 5, 7 represent different days).

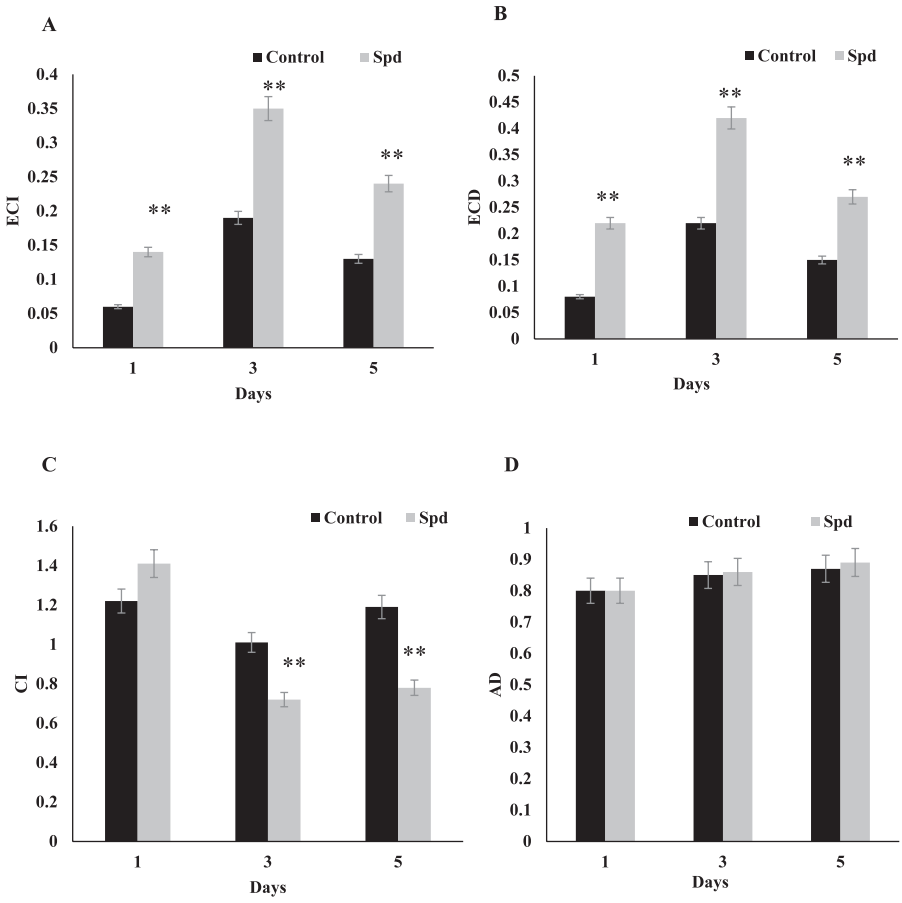

Fig. 3. Nutrition indices of control and spermidine-treated larvae. (A) CI, (B) AD, (C) ECI, and (D) ECD. **Highly significant ($P < 0.01$) in comparison with control.

in the gut tissue. The FRAP assay revealed that spermidine treatment significantly increased ($t = 2.20$; df = 11; $P = 0.008$) the antioxidant activity of the gut sample on days 1, 3, and 7 compared with the control (Fig. 5A). Moreover, the DPPH and SOD assays ($t = 2.20$; df = 11; $P < 0.00001$) confirmed the enhanced inhibition of free radicals after spermidine consumption (Fig. 5B, C). Our results thus support the idea that spermidine administration enhances the ability of free radical scavenging in the *B. mori* gut, thereby reducing oxidative stress and improving metabolism.

Cell viability response. Increased antioxidant activity is positively correlated with cell viability. The MTT assay was performed with gut samples from control and spermidine-treated larvae on days 1, 3, 5, and 7. The metabolic activity of gut samples increased from day 1 to day 5 and decreased toward day 7, as larvae consumed more food from day 1 to day 5, and food consumption decreased after

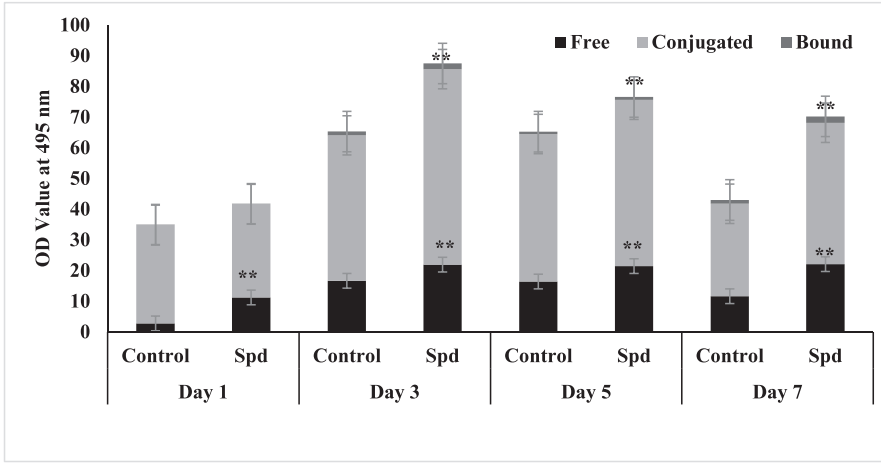

Fig. 4. Intracellular polyamine levels in the gut tissue of control and spermidine-fed *B. mori* larvae.

day 5. The spermidine-treated group exhibited a significant increase ($t = 2.20$; df = 11; $P = 0.003$) in metabolic activity on days 3 and 5 compared with the control group (Fig. 6). This validates the role of spermidine supplementation in improved and efficient utilization of the diet compared with control larvae.

Gut bacteriota response. Nutrition, metabolic activity, and gut microbiota are interconnected (Ayayee et al. 2018, Chen et al. 2016, Visôtto et al. 2009). Bacteriota from the guts of control and spermidine-treated larvae were analyzed on day 5 by the culture-dependent method. The total cultivatable bacterial count in the spermidine-treated gut sample was 3.73×10^{10} CFU/mL, which was significantly ($t = 3.18$; df = 3; $P = 0.001$) greater than that of the control group with 2.78×10^{10} CFU/mL (Fig. 7). Bacterial isolates that were common to both the control and spermidine-treated group were *Escherichia coli* Migula 1895*, Pseudomonas, Erwinia, Enterococcus*, and *Enterobacter*. The control group exclusively harbored *Serratia, Acinetobacter*, and *Klebsiella*, whereas the spermidine-treated group specifically included *Staphylococcus, Bacillus cereus* Frankland and Frankland 1887, *Citrobacter, Lactobacillus, Micrococcus,* and *Aeromonas* (Table 1). The predominant genera from the control groups belonged to the *Proteobacteria*. In contrast, the predominant genera from the spermidine-treated group was *Firmicutes* (Turnbaugh et al. 2006). We speculate that the overall increase in the bacteriota in the guts of spermidine-fed larvae could be due to the changes in its gut microbiota, which enhance nutritional uptake and breakdown.

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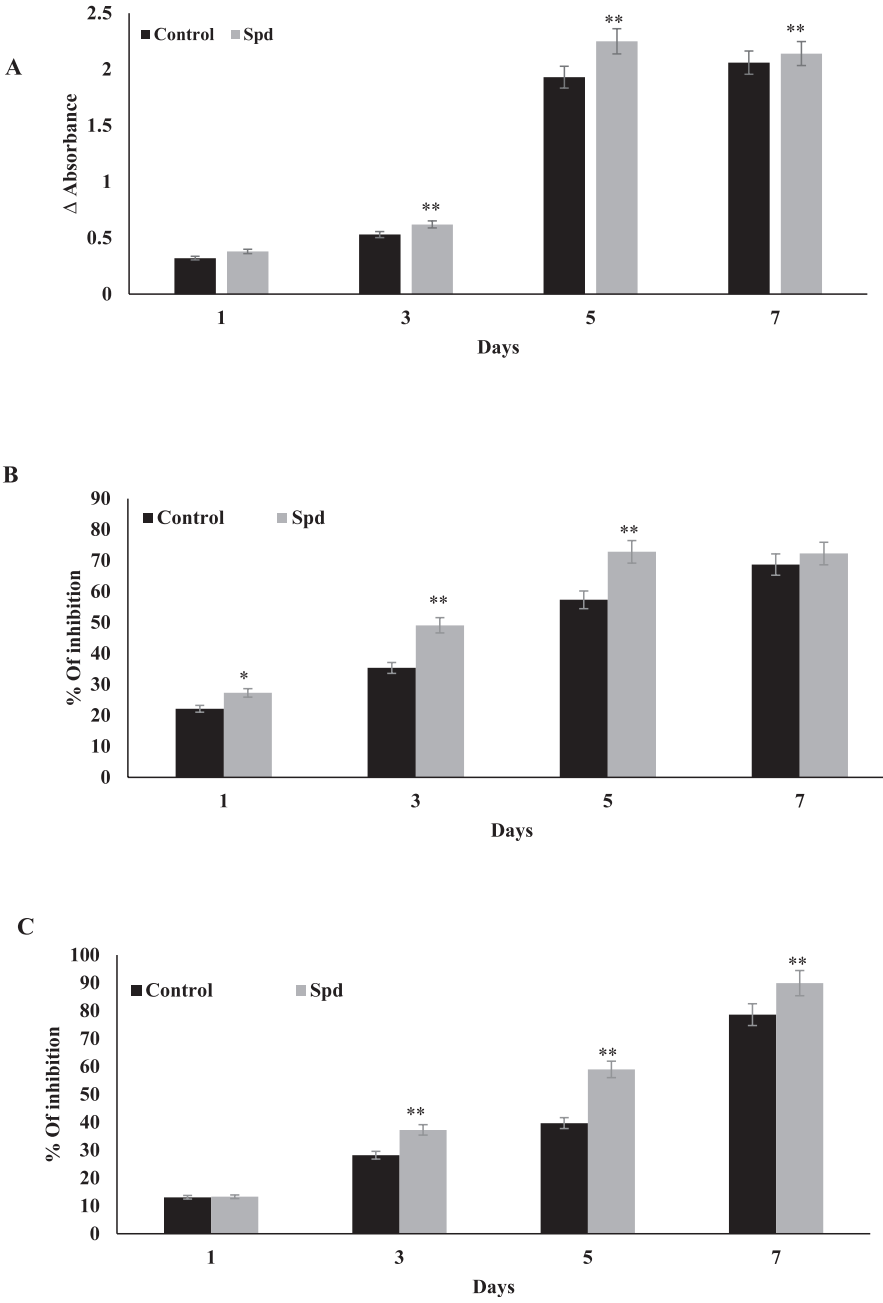
Fig. 5. Antioxidant analysis of gut tissue during fifth-instar larval development. (A) FRAP assay of control and spermidine-treated larvae (*y* axis represents change in absorbance at 593 nm). (B) DPPH assay of



control and spermidine-treated larvae (*x* axis denotes days and *y* axis represents percentage of inhibition). (C) SOD assay (*x* axis denotes days and *y* axis represents percentage of inhibition).

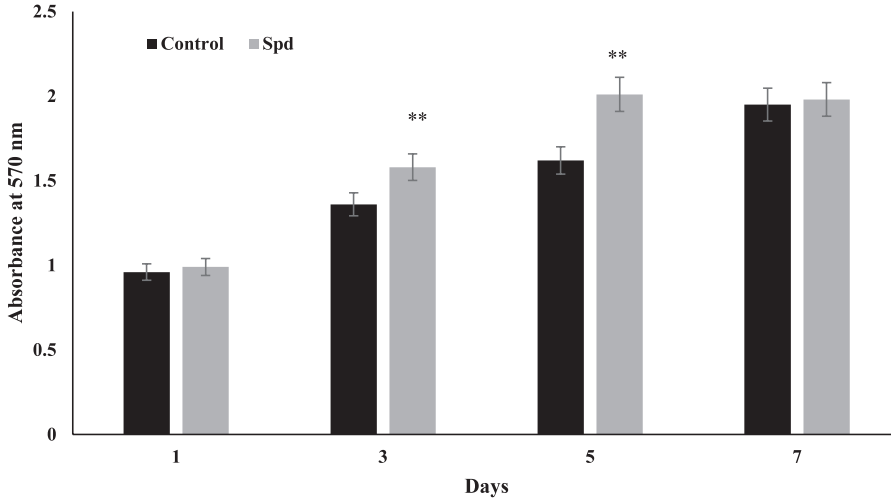


Fig. 6. Cell viability (MTT) assay of gut tissues of control and spermidine-treated *B. mori* larvae.

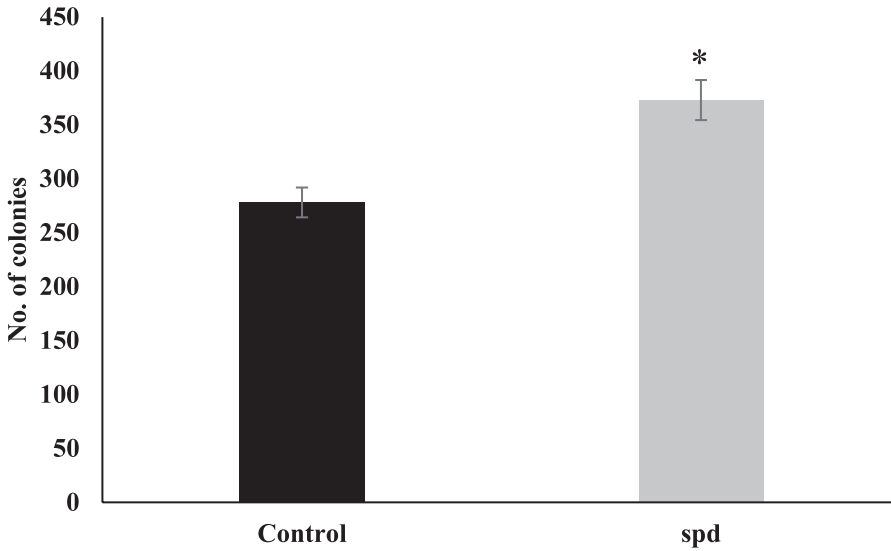


Fig. 7. Pure culture colonies isolated from gut tissues of control and spermidine-treated *B. mori* larvae. *Significant ($P < 0.05$).

Table 1. Bacterial isolates identified from gut tissues of control and spermidine-fed *B. mori* fifth-instar larvae.

Isolates	Organism	Control	Spd
1	*Staphylococcus aureus*	−	+
2	*Bacillus cerus*	−	+
3	*Citrobacter*	−	+
4	*Pseudomonas*	+	+
5	*Lactobacillus*	−	+
6	*Enterobacter*	+	+
7	*Enterococcus*	+	+
8	*Acinetobacter*	+	−
9	*Serratia*	+	−
10	*Micrococcus*	−	+
11	*Escherichia coli*	+	+
12	*Erwinia*	+	+
13	*Aeromonas*	−	+
14	*Klebsiella*	+	−

Discussion

Insect performance is associated with food consumption and utilization (Slansky 1990). The importance of diet in producing nutritionally efficient silkworms for proper growth and development has long been studied (Murugan and George 1992). The growth and development of an organism is influenced by its ability to convert nutrients into energy and body mass (Sogbesan and Ugwumba 2008). The chemical composition of the host plant contributes to the insect's survival, growth, and development (Pullin and Martin 2004, Samraj and David 1988, Sharma et al. 1982). A combination of plant characteristics may be responsible for insect preference and performance, and an optimal combination of plant components serves to maximize host suitability (Foss and Rieske 2003). Increased larval weight might induce developmental changes like onset of prepupal stage and extrusion of silk for cocoon formation as previously described in *Drosophila melanogaster* Meigen 1830 (Boulan et al. 2015, Scott et al. 2004, Warren et al. 2006). In our study, the spermidine-fed group showed a significant increase in the ECI index, which could have contributed to its increased growth (Senthil Nathan et al. 2005), and the significant increase in the ECD index helped in maintaining the proportion of digested food and its conversion into energy (Naseri et al. 2010). Numerous studies on *Helicoverpa armigera* (Hubner) and other insects showed that high ECI and ECD values are frequently associated with low CI values (Barbosa and Greenblatt 1979,

Hemati et al. 2012). We observed similar results in the spermidine-treated groups due to the satiety of larvae on consumption of spermidine fortified diet.

Digestive tracts absorb a considerable amount of polyamine introduced from food (Matsumoto et al. 2012). In addition, in rats, polyamine is important for the growth and development of the digestive tract wall (Löser et al. 1999). Our results mirrored these observations, where polyamine levels increased in gut tissue from day 1 to day 3 and decreased later during fifth-instar development. Furthermore, consistent with our findings, supplementation with spermidine has been reported to increase endogenous polyamine levels and enhance the growth of *Saccharomyces cerevisiae* Hansen 1883, *D. melanogaster*, *Caenorhabditis elegans* Maupas 1900, and cultured human immune cells (Eisenberg et al. 2009).

Naturally occurring polyamine acts as a free radical scavenger to protect DNA and phospholipids from oxidative stress (Ha et al. 1998, Khan et al. 1992). Gut samples from spermidine-fed larvae showed enhanced free radical scavenging and reduced potential that helps in increasing the metabolic activity of gut tissue, as shown in yeast, rat, zebrafish larvae, and *D. melanogaster* (Jeong et al. 2018, Minois et al. 2014).

The gut microbiome plays an important role in utilizing nutrition (Chen et al. 2018). Studying the changes in microbiomes after different nutritional supplements will help determine an optimal diet for *B. mori*. The abundance and diversity of gut microbiome of spermidine-fed *B. mori* larvae increased significantly over that of the control group. Our data comparing intrinsic gut bacteria between control and spermidine-treated larvae agree with earlier studies (He et al. 2013, Sun et al. 2016, Vitthalrao and Rajendra 2012, Xiang 2007). The *Proteobacteria* symbionts present in both control and spermidine groups aid in degrading polymers present in mulberry leaves to provide nutrients (Sun et al. 2016), whereas gut *Firmicutes* increase the energy harvest from the diet (Turnbaugh et al. 2006). Spermidine promoted the growth of a beneficial microbiome in the gut tissue as seen in neonatal mice (Gómez-Gallego et al. 2014). In addition, our observations were consistent with another study conducted by Rajan et al. (2020) where bacterial diversity and richness were observed in bacterial profiles from guts of spermidine-fed larvae compared with controls.

The present study describes the effect of spermidine treatment on gut growth of *B. mori* larvae. Our results highlight the potential use of spermidine to enhance nutritional indices and to establish a framework for future studies on improving the nutritional diet for *B. mori* to increase silk production.

Acknowledgments

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