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Lectin-Producing Bacteria from Silkworm (*Bombyx mori*) Gut Microbiota: Isolation, Characterization, and Biofunctional Analysis

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Abstract

Lectins are carbohydrate-binding proteins with antimicrobial, antioxidant, and immunomodulatory properties. Microbial lectins are increasingly recognized as sustainable substitutes for plant-derived lectins because of their straightforward culture and scalable synthesis; yet, lectin-producing bacteria inside the gut microbiome of insects, especially in the silkworm (*Bombyx mori*), remain largely unexplored. This study aimed to isolate, characterize, and evaluate the bio-functional properties of lectin-producing bacteria from the gut of fifth-instar *B. mori* larvae. Gut-associated isolates were evaluated for lectin production through a hemagglutination assay, resulting in two lectin-positive isolates identified by 16S rRNA sequencing as *Enterococcus faecium* NBRC 100486 and *Enterococcus faecium* DSM 20477, both exhibiting 99.13% similarity to reference strains. The partially purified lectins were obtained by ammonium sulfate precipitation, with the best yield at 65% saturation, at protein concentrations of 2.69 mg/mL and 2.08 mg/mL. Hemagglutination activity was highest at 25 °C and decreased at higher temperatures, suggesting intermediate thermal stability. The carbohydrate inhibition assay showed high carbohydrate specificity, with the highest inhibition by lactose, followed by sucrose, glucose, and fructose. Crude extracts had no antibacterial action alone, but metal ions considerably boosted it, especially against *Bacillus subtilis*, suggesting a metal-dependent change of lectin function. The lectins demonstrated significant antioxidant activity, with DPPH radical-scavenging efficiencies of 68.8% and 73.8%. The results obtained in this study have shown that *E. faecium* isolated from the gut of silkworms can be a potential source of bioactive lectins that show carbohydrate-binding properties, antioxidant, and metal-enhanced antibacterial properties relevant to biomedical and biotechnological applications.

Keywords: *Bombyx mori*, hemagglutination, lectin-producing bacteria, *Enterococcus faecium*

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1. Introduction

Lectins have traditionally been isolated from plants, particularly from the seeds of leguminous plants [1],[2]. However, microbial sources are now deemed more beneficial, as microorganisms are simpler to cultivate, exhibit rapid growth, and can be genetically modified [3]. Microorganisms have also been found to be a reliable and promising source of lectins, as evidenced by the presence of lectin-like proteins with considerable bio-functional ability in bacteria like *Acinetobacter baumannii* and *Lactobacillus acidophilus* [3],[4]. Besides free-living microorganisms, those associated

with hosts, such as those found in insect guts, have also been recognized as a valuable and under-explored source of bioactive metabolites, including novel antimicrobial and antiproliferative compounds [5]. Insects, therefore, are an important, yet underexploited source for the discovery of microbial lectins of medicinal interest.

Bombyx mori (silkworm) is an economically important insect that has been domesticated for thousands of years in silk production and is a well-established genetic and biological model organism. The organism has a clearly defined life cycle, well-controlled rearing conditions, and is economically important, making it an ideal organism for physiology and microbiology studies [6]. Other than silk production, the silkworm has been the subject of scientific research because of its immune system, digestive physiology, and ability to digest polysaccharides owing to the bacteria found in its gut [7]. In the resource-rich ecological niche of the silkworm gut, microbial communities are engaged in host defense and metabolic processes of nutrition and digestion [8].

Insect gut bacteria are becoming recognized as a diverse group of metabolically active bacteria. The gut microbiome of silkworms facilitates the digestion of mulberry leaves, the synthesis of vital nutrients, and provides defense against harmful organisms [9]. Insects' gut microorganisms have been shown to synthesize a wide variety of bioactive molecules like enzymes, secondary metabolites, and antimicrobial peptides [10], which can support host survival and immunity. Although the gut microbiota of insects is important for their ecology and biology, few studies have focused on isolating lectin-producing bacteria from the guts of insects. This area could be explored for the discovery of new strains of bacteria that may be able to produce lectins with antioxidant and antibacterial properties. Addressing this gap necessitates meticulous consideration of developmental time, as the composition and metabolic activity of the silkworm gut microbiota are known to vary across larval instar stages [11]. The life cycle of the silkworm can be divided into five larval stages (instars) and the intervening moulting stages. The fifth instar is the most vigorous feeding stage before pupation, and the gut bacteria are most numerous and active at this stage [11]. This developmental stage was selected for the ongoing study because it might be best suited for restoring the gut bacterial community with a diversified and metabolically active community, including the ability to produce lectins.

The objective of this study was to isolate and identify lectin-producing bacteria from the gastrointestinal tract of fifth-instar *Bombyx mori* larvae, assess the isolates for antibacterial and antioxidant properties, partially purify the lectin proteins using molecular techniques, and evaluate their lectin activity. This study not only emphasizes silkworms as a possible source of bioactive lectins but also enhances the understanding of the variety within the silkworm gut microbiome.

2. Materials and Methods

2.1. Insect collection and sample preparation

Fifth-instar silkworm larvae were procured from the Rajshahi sericulture factory in Bangladesh and delivered to the laboratory in sterile containers. The larvae were surface-sterilized by immersion in 70% ethanol for 1-2 min to eradicate external bacteria, followed by multiple rinses with sterile phosphate-buffered saline (PBS) to remove excess ethanol. The surface-sterilized fifth-instar larvae were subsequently dissected, and the whole gut was meticulously excised and homogenized in PBS using a sterile mortar and pestle. The homogenate served as the raw material for isolating gut-associated bacteria.

2.2. Isolation of microorganisms from gut contents

The obtained gut homogenate was inoculated onto De Man, Rogosa, and Sharpe (MRS) agar plates and incubated at 37 °C for 24-48 h under anaerobic conditions. Bacterial colonies with distinct morphological traits were selected and purified through successive subculturing via the streak plate method. The purification process was repeated multiple times until a uniform colony shape was obtained, thereby validating the achievement of pure cultures. The purified isolates were subsequently preserved at -80 °C for long-term storage in 50% glycerol stocks.

2.3. Screening for lectin activity

Lectin activity was evaluated following a hemagglutination test with slight modifications [12]. Purified bacterial isolates were grown in MRS broth at 37 °C for 24 h and subsequently centrifuged at 4000 rpm for 15 min to obtain cell-free supernatant, which functioned as the crude lectin extract. A 2% (v/v) suspension of red blood cells (RBCs) was formulated in PBS. Equal volumes of the crude lectin extract and RBC suspension were added to a sterile 96-well microtiter plate, with phosphate-buffered saline used as the negative control. Following gentle mixing and a 60 min incubation at room temperature, hemagglutination was evaluated visually, and lectin-positive isolates were selected for molecular identification.

2.4. Molecular identification

Genomic DNA was extracted from an individual colony suspended in 20 µL of TE buffer. The suspension was heated to 95 °C for 10 min in a thermocycler, followed by centrifugation at 6,000 rpm for 2-3 min. The transparent supernatant served as the DNA template. The 16S rRNA gene was amplified by PCR with universal primers 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1510R (5′-GGCTACCTTGTTACGA-3′) [13]. The reaction started with an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation (94 °C for 1 min), annealing (50 °C for 1 min), and extension (72 °C for 1.5 min). The final extension phase was conducted at 72 °C for 5 min. The amplification of the 16S rRNA gene was verified using a 0.8% agarose gel. PCR products were dispatched to icddr,b in Dhaka, Bangladesh, for Sanger sequencing. The acquired sequences were evaluated in comparison with the accessions in the National Center for Biotechnology Information database (NCBI) using the BLASTn search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Crude lectin extraction and partial purification

Overnight-grown lectin-producing bacterial isolates were centrifuged at 4000 rpm for 15 min to obtain cell-free supernatant, which served as the source of extracellular lectin. Proteins were partially purified using ammonium sulfate precipitation (40-80% saturation) with continuous agitation at low temperature, followed by a 1 h incubation to achieve full precipitation [14]. The precipitated proteins were harvested via centrifugation at 10,000 rpm for 15 min, resuspended in PBS, and used as the partly purified lectin preparation for further investigations. The preparation was maintained at 4 °C until subsequent analysis.

2.6. Protein concentration measurement by Bradford assay

The protein concentrations of crude Lectin-2 and Lectin-3 were quantified following the Bradford protein assay [15]. In summary, 100 µL of Bradford reagent was combined with 10 µL of distilled water that served as a blank. Subsequently, 100 µL of Bradford solution was combined with 10 µL of crude Lectin-2 and Lectin-3 samples separately. Samples exhibiting a dark blue hue were diluted (10-, 50-, or 100-fold) with distilled water and re-evaluated until a light blue hue, signifying the measurable range, was achieved. Absorbance was quantified at 595 nm using a UV-Vis spectrophotometer, adhering to an acceptable optical density (OD) range of 0.02-0.16. The blank absorbance was deducted from the sample absorbance to derive the final OD. All measurements were conducted in triplicate.

2.7. Effect of temperature on hemagglutination activity

The effect of temperature on lectin-mediated hemagglutination activity was evaluated using a hemagglutination assay [12]. The reaction mixture was prepared as previously described. The reaction mixtures were incubated at three different temperatures: room temperature, 37 °C, and 45 °C. Incubation was carried out for 1 hour under controlled conditions. After incubation, hemagglutination was assessed visually and spectrophotometrically by measuring the absorbance at 540 nm. Each experiment was performed in triplicate to ensure reproducibility, and the mean OD values were calculated.

2.8. Carbohydrate inhibition assay

A carbohydrate inhibition assay was performed to ascertain the carbohydrate-binding specificity of lectins produced by the selected bacterial isolates (Isolate-2 and Isolate-3). Glucose, fructose, lactose, and sucrose (100 mM in sterile PBS) served as the experimental carbohydrates. Three controls were incorporated: a negative control (PBS + RBC), a positive control (lectin + PBS + RBC), and a carbohydrate control (carbohydrate + PBS + RBC) to validate the assay. Equal amounts of crude lectin extract and each carbohydrate solution were combined and pre-incubated at room temperature for 30 min. Thereafter, an equivalent volume of 2% (v/v) RBC suspension formulated in PBS was introduced into each well of a sterile 96-well U-bottom microtiter plate. The mixes were gently added and incubated at room temperature, following which hemagglutination was evaluated visually and by measuring the optical density (OD) at 540 nm with a microplate reader. All experiments were conducted in triplicate, and the average OD values were calculated [16].

2.9. Antimicrobial activity test

The antibacterial efficacy of crude Lectin-2 and Lectin-3 was assessed against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Shigella* spp. with the agar well diffusion technique with minor modifications [17]. The selected pathogenic strains were cultured in LB broth at 37 °C for 18-24 h and spread onto sterile Mueller–Hinton agar (MHA). 6 mm in diameter wells were created using a sterile cork borer, and 50, 100, 150, or 200 µL of crude lectin was introduced into the respective wells. After incubation at 37 °C for 18-24 h, the antibacterial activity was evaluated by measuring the zones of inhibition (mm). Furthermore, 5 mM solutions of $CaCl_2$, NaCl, KCl, $MnSO_4 \cdot H_2O$, and EDTA were prepared to assess the effects of metal ions on the antimicrobial activity of lectins. Crude lectin from each sample was individually mixed with each solution and incubated at room temperature for 30 min before 200 µL of the mixture was dispensed into wells on

MHA. Following incubation at 37 °C for 18-24 h, the zones of inhibition were quantified and compared with those generated by the crude lectin alone.

2.10. Antioxidant activity test

The antioxidant efficacy of the lectin was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay with minor modifications [18]. A 0.02 mM DPPH solution was prepared in methanol. Lectin samples (100, 200, and 300 µL) were diluted to a final volume of 1 mL with methanol, thereafter supplemented with 3 mL of DPPH solution. The reaction mixtures were incubated in the dark at room temperature for 30 min, with a control comprising methanol and DPPH without lectin serving as the reference. Absorbance was quantified at 517 nm using a UV-Vis spectrophotometer, employing methanol as the blank solution. The DPPH radical scavenging activity was determined as follows:

Radical scavenging activity (%) = $[(A_0 - A_t) / A_0] \times 100$

A_0 represents the absorbance of the control, while A_t denotes the absorbance of the lectin sample. All assays were conducted in triplicate [19].

3. Results

3.1. Isolation and screening of lectin-producing bacteria

Bacterial isolates were obtained from the intestinal homogenate of surface-sterilized fifth-instar silkworm larvae after aseptic dissection. Distinct bacterial colonies were isolated using repeated streaking and evaluated for lectin activity via a hemagglutination assay. Lectin activity was assessed by analyzing the agglutination pattern of red blood cells (RBCs). Isolate-2 and Isolate-3 demonstrated widespread mat formation across the wells, signifying positive hemagglutination, but Isolate-1 produced a compact red button at the wells' bottom, suggesting absence of lectin activity **(Figure 1)**. Based on these findings, Isolate-2 and Isolate-3 were selected for further characterization.

Figure 1. Screening of lectin-producing bacterial isolates by hemagglutination assay. Isolate-2 and isolate-3 showed positive hemagglutination, whereas isolate-1 showed no hemagglutination activity.

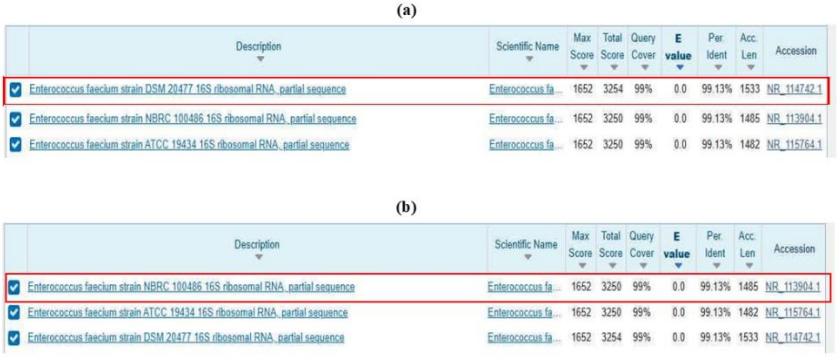

Figure 2. Molecular identification of lectin-positive bacterial isolates based on 16S rRNA gene sequencing. Isolate 1 was identified as *Enterococcus faecium* NBRC 100486, while isolate 2 was identified as *Enterococcus faecium* DSM 20477.

3.2. Molecular identification of positive isolates

The lectin-positive isolates were subjected to molecular identification by 16S rRNA gene sequencing. The amplified gene sequences were analyzed using BLAST against the NCBI database. The sequence of isolate-2 showed 99.13% similarity with *Enterococcus faecium* DSM 20477. Similarly, isolate-3 showed 99.13% similarity with *Enterococcus faecium* NBRC 100486 **(Figure 2)**.

3.3 Protein concentration measurement by Bradford assay

Crude lectin proteins were precipitated using ammonium sulfate at different saturation percentages. Among the tested concentrations, 65% ammonium sulfate saturation showed the highest protein yield for both isolates. At 65% saturation, the protein concentration of Lectin-2 was 2.69 mg/mL, while Lectin-3 showed 2.08 mg/mL **(Figure 3)**. These

results indicate that 65% ammonium sulfate saturation was optimal for lectin precipitation from both isolates. Furthermore, Lectin-2 exhibited a comparatively higher protein yield than Lectin-3 under the same precipitation condition.

3.4. Effect of temperature on hemagglutination activity

The stability of lectin activity under different temperature conditions was evaluated by performing a hemagglutination assay at varying temperatures. The optimum hemagglutination activity was observed at 25 °C, indicating that this is the optimum temperature for lectin functionality **(Figure 4)**. At temperatures above 25 °C, a gradual decrease in agglutination activity was observed.

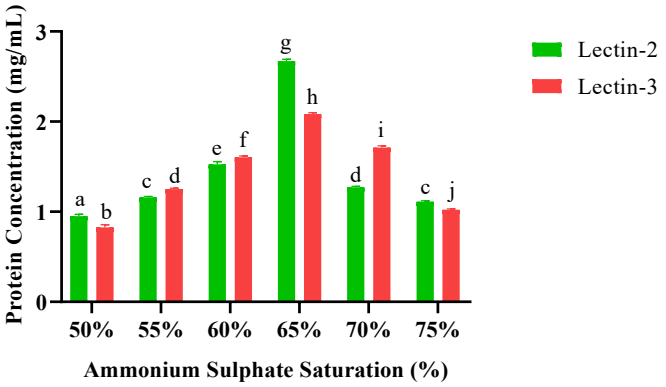

Figure 3. Measurement of protein concentration in crude lectins by Bradford assay. The experiment was performed in triplicate (n = 3), and data are presented as mean ± SD. Different lowercase letters indicate significant differences among treatments (Tukey's HSD, $P < 0.05$).

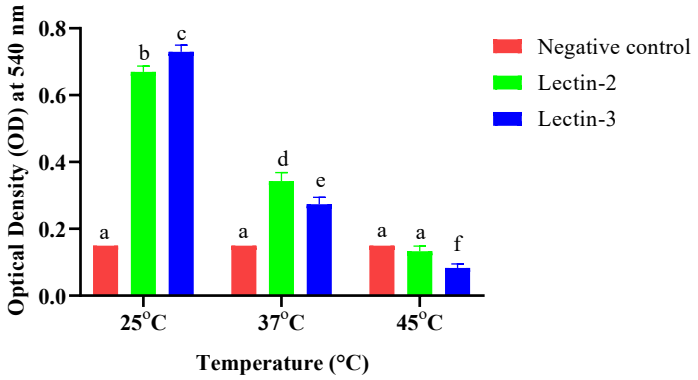

Figure 4. Temperature dependence of the hemagglutination activity of crude lectins. Data are presented as mean ± SD (n = 3). Bars sharing the same lowercase letter are not significantly different (Tukey's HSD, $P < 0.05$).

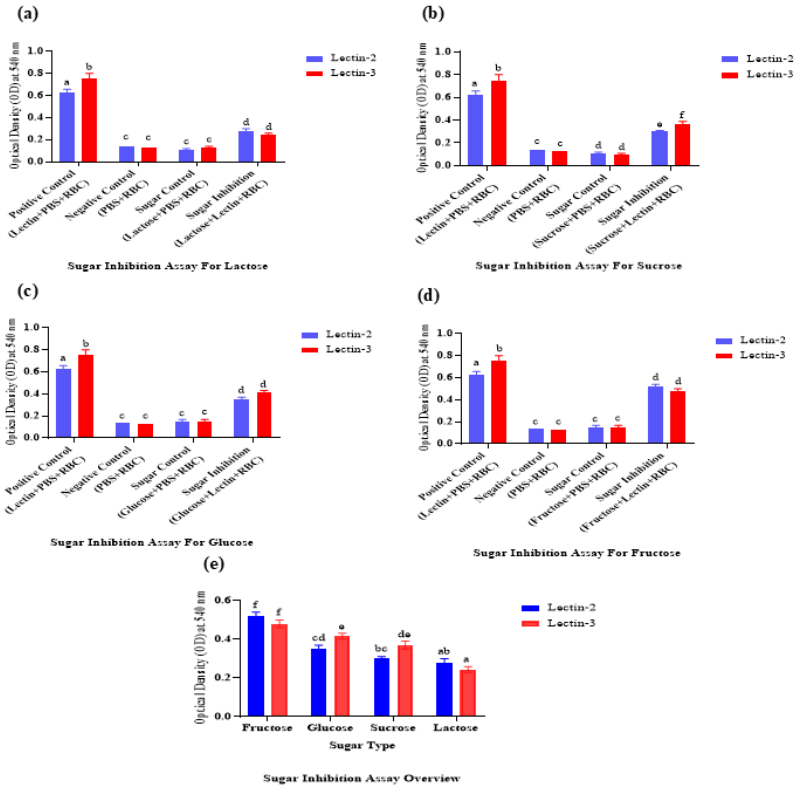

Figure 5. The inhibition of lectin-mediated hemagglutination by (a) lactose, (b) sucrose, (c) glucose, (d) fructose, and (e) comparative overview of the carbohydrate inhibition assay. The experiment was carried out in three replicates (n=3), and the results are expressed as mean ± SD. Different lowercase letters above the bars indicate significant differences among the lectin × sugar combinations according to two-way ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test ($P < 0.05$). Bars sharing the same lowercase letter are not significantly different.

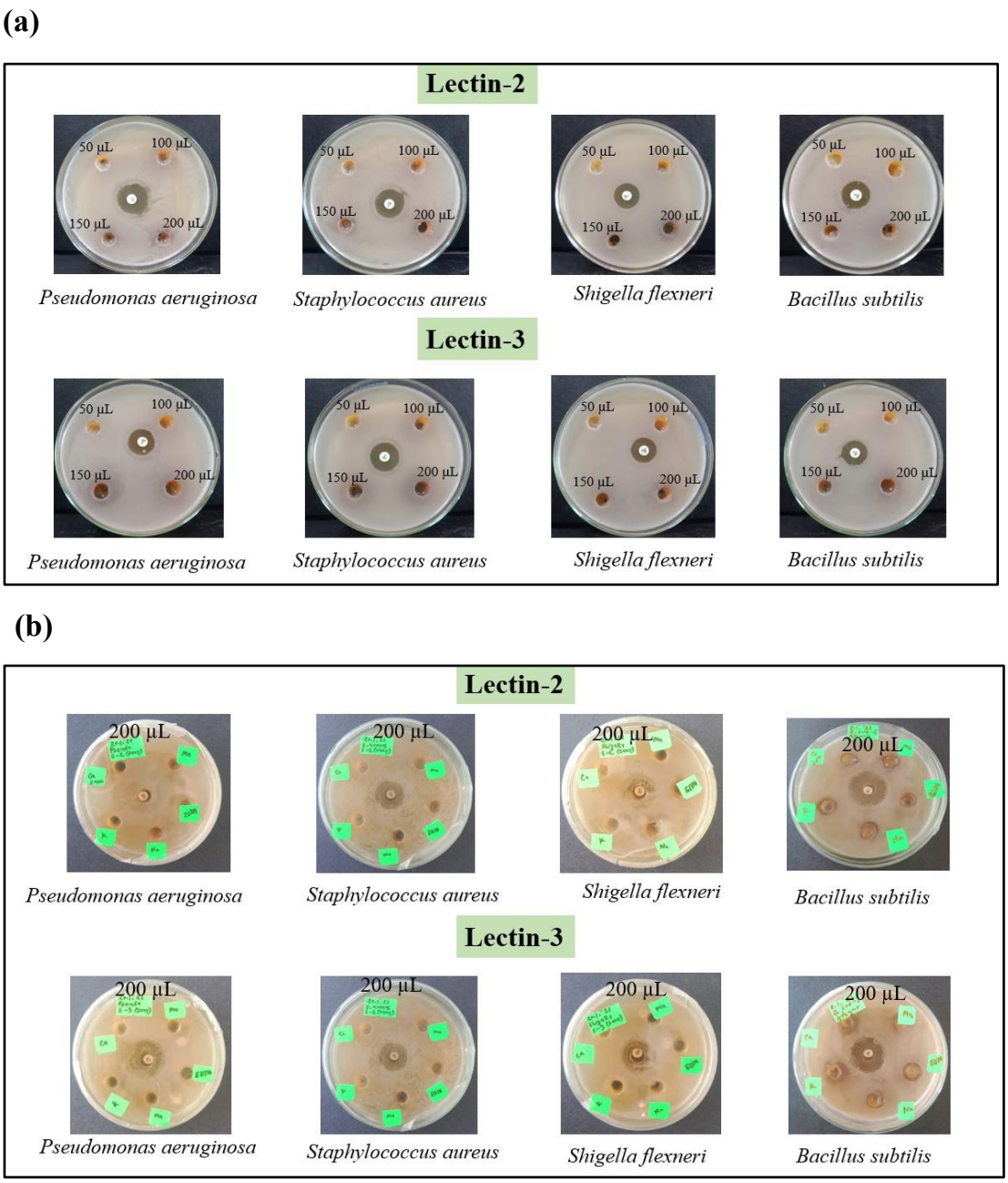

Figure 6. Antimicrobial activity of crude lectins against selected pathogenic bacteria in the absence (a) and presence (b) of metal ions.

3.5. Carbohydrate inhibition assay

Glucose, fructose, sucrose, and lactose were assessed individually for their capacity to prevent hemagglutination. Of the carbohydrates studied, lactose demonstrated the most significant inhibitory impact, suggesting the highest binding affinity of the lectins for lactose residues **(Figure 5a)**. Sucrose exhibited modest inhibition **(Figure 5b)**, followed by glucose and fructose **(Figure 5c & 5d)**. The carbohydrate-binding specificity of the lectins was ranked as lactose > sucrose > glucose > fructose, demonstrating a preferential affinity for lactose **(Figure 5e)**.

3.6. Antimicrobial activity of crude lectins

The antibacterial efficacy of crude lectin was assessed against four pathogenic strains using the agar well diffusion assay **(Figure 6)**. No antibacterial action was detected when only the crude lectins were used. The incorporation of metal ions (Ca^{2+}, Na^{+}, K^{+}, Mn^{2+}, and EDTA) modified the antibacterial efficacy of the lectins. Lectin-2 demonstrated a significant inhibitory effect against *B. subtilis* in the presence of K^{+} and Na^{+}, while only modest inhibition was noted with Ca^{2+}, Mn^{2+}, and EDTA **(Table 2)**. Conversely, Lectin-3 exhibited moderate action with Ca^{2+}, K^{+}, and EDTA, and little activity with Na^{+}

and Mn^{2+} **(Figure 7)**. However, ciprofloxacin (positive control) generated significantly wider zones of inhibition compared to both lectin preparations **(Table 1)**.

3.7. Antioxidanta of crude lectins

The antioxidant property of the crude lectin extracts was determined using the percentage inhibition of free radicals. Lectin-2 showed 68.8% antioxidant activity, whereas Lectin-3 showed 73.8% activity **(Table 2)**.

Table 1. Antimicrobial activity of crude Lectin-2 and Lectin-3 in the presence of 5 mM metal ions and EDTA against *Bacillus subtilis*. Data are presented as mean ± SD (n = 3).

Treatment	Zone of inhibition (mm)	Interpretation	Zone of inhibition (mm)	Interpretation
	Lectin-2		Lectin-3	
Ciprofloxacin	22.0 ± 1.25	Strong	25±1.65	Strong
Ca^{2+}	11.0 ± 0.99	Weak	16±1.54	Moderate
K^{+}	16.0 ± 1.16	Moderate	17±1.29	Moderate
Na^{+}	15.0 ± 1.31	Moderate	12±0.98	Weak
Mn^{2+}	11.0 ± 1.22	Weak	10±0.99	Weak
EDTA	10.0 ± 1.45	Weak	15±1.11	Moderate

Table 2. The results of the antioxidant test of the lectins. The experiment was conducted three times (n = 3), and the data are shown in terms of mean ± SD.

Sample Name	Inhibition (%)
Lectin-2	68.8% ± 3.26
Lectin-3	73.8% ± 4.21

4. Discussion

Lectins are carbohydrate-binding proteins that recognize specific sugar structures without altering their own chemical composition. They are now known to occur across nearly all groups of living organisms, where they support cell-cell communication, pathogen recognition, and immune defense [20],[21]. The hemagglutination assays in this study confirmed the lectin activities of both Lectin-2 and Lectin-3, which is consistent with the classical use of agglutination of red blood cells as a diagnostic marker for lectin activity. Carbohydrate inhibition assays indicated that the binding specificity of the lectin was sugar-specific and that the lectin showed a higher affinity towards lactose as compared to glucose, sucrose, and fructose, similar to that of other lectins and consistent with the binding specificity of other microbial lectins characterized [22],[23].

The most efficient precipitation of Lectin-2 and Lectin-3, with maximum yield at 65%, was within the 40-70% range reported for proteins of intermediate hydrophobicity [24]. This is important because this condition would be optimal for protein concentration with the purging of impurities to a minimum. This conforms to the purification approach used for other bioactive lectins, including a recently characterized galactose-binding seed lectin also isolated via ammonium sulfate precipitation [25].

The purified lectins inhibited the growth of the Gram-positive bacterium *Bacillus subtilis*, presumably by binding to glycans on the bacterial surface recognized by peptidoglycan and teichoic acid, which may lead to destabilization of the cell wall and/or affect adhesion. This is similar to the antimicrobial activity of lectins from plants, microorganisms, and insects reported by Konozy et al., 2022 [26] and a similarly purified plant seed lectin, which also demonstrated higher bacteriostatic activity against Gram-positive than Gram-negative bacteria, indicating Gram-positive selectivity may be a common, but not universal, characteristic of carbohydrate-specific lectins from a variety of biological sources [25],[27].

This activity was further enhanced by metal ions (Na^{+}, K^{+}, Ca^{2+}), consistent with the requirement of many lectins, particularly C-type lectins, to stabilize their carbohydrate-recognition domain (CRD) [27],[28]. It is this stabilization of the CRD that most plausibly explains the improved binding efficiency observed here.

Lectin-2 and Lectin-3 also displayed notable free-radical scavenging activity, with DPPH inhibition of 68.8% and 73.8%, respectively, consistent with reports attributing antioxidant activity in plant and microbial lectins to electron-donating amino acid residues [29],[30]. Notably, these values exceed those reported for a comparable galactose-binding seed lectin, which showed only 42.83% DPPH scavenging at a similar concentration, suggesting the present lectins possess comparatively strong antioxidant potential even at the partially purified stage [25].

The antimicrobial and antioxidant functions are part of the overall ecological function of the gut microbiome of the silkworm, which is gaining growing attention as a complex ecosystem that plays a role in the host's immune system and digestion. The presence of lectin-producing bacteria in this niche supports the hypothesis that symbiotic microorganisms contribute to host defense, potentially complementing the insect's own innate immune lectins.

The present study was limited to crude and partially purified extracts; further purification, molecular and structural characterization, and *in vivo* evaluation will be required to define the precise lectin types, mechanism of action, and therapeutic safety and efficacy. Overall, the findings identify lectins from silkworm gut-associated bacteria as promising candidates for antimicrobial and antioxidant applications in biomedical research.

5. Conclusion

In this study, lectin-producing bacteria were isolated from the gut of fifth-instar *Bombyx mori* larvae and were identified as *Enterococcus faecium* by the sequence similarity of the 16S rRNA gene with reference strains. Isolate-2 showed 99.13% similarity with DSM 20477, whereas isolate-3 showed 99.13% similarity with NBRC 100486. Lectin-2 and Lectin-3 were partially purified by ammonium sulfate precipitation, in which 65% saturation showed 2.69 mg/mL and 2.08 mg/mL of proteins, respectively. Functional characterization indicated that the highest hemagglutination was observed at 25 °C and high carbohydrate specificity, with lactose having the highest inhibition. Lectin extracts both showed moderate antimicrobial activity against *Bacillus subtilis*, and this activity increased in the presence of metal ions, suggesting metal-dependent antimicrobial activity. Furthermore, both lectins displayed significant antioxidant activity, with DPPH radical-scavenging efficiencies of 68.8% and 73.8% for Lectin-2 and Lectin-3, respectively. In conclusion, the results presented here suggest a promising source of bioactive lectins for antimicrobial and antioxidant therapeutics from the gut microbiota of silkworms.

Author contributions: Conceptualization: UAN, S.Z., and M.A.S.; methodology: UAN, DDH, RT, MA; validation: MAS and SZ; formal analysis: UAN, DDH, TA, MSM; investigation: UAN, DDH, TA, MA, MSM; resources: MAS, and SZ; writing original draft preparation: UAN, DDH, TA, MA; writing review and editing: MAS, and SZ; supervision: MAS, and SZ; project administration: MAS, and SZ. All authors have read and agreed to the published version of the manuscript.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests: The author declares no conflict of interest.

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Ethical approval: The manuscript does not contain experiments using humans and animals.

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