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Original Research

Chitinase Producing Gut-Associated Bacteria Affected the Survivability of the Insect *Spodoptera frugiperda*

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Abstract

Background: Fall armyworm (*Spodoptera frugiperda*) is a highly destructive maize pest that significantly threatens agricultural productivity. Existing control methods, such as chemical insecticides and entomopathogens, lack effectiveness, necessitating alternative approaches. **Methods**: Gut-associated bacteria were isolated from the gut samples of fall armyworm and screened based on their chitinase and protease-producing ability before characterization through 16S rRNA gene sequence analysis. The efficient chitinase-producing *Bacillus licheniformis* FGE4 and *Enterobacter cloacae* FGE18 were chosen to test the biocontrol efficacy. As their respective cell suspensions and extracted crude chitinase enzyme, these two isolates were applied topically on the larvae, supplemented with their feed, and analyzed for their quantitative food use efficiency and survivability. **Results**: Twenty-one high chitinase and protease-producing bacterial isolates were chosen. Five genera were identified by 16S rRNA gene sequencing: *Enterobacter*, *Enterococcus*, *Bacillus*, *Pantoea*, and *Kocuria*. In the biocontrol efficacy test, the consumption index and relative growth rate were lowered in larvae treated with *Enterobacter cloacae* FGE18 by topical application and feed supplementation. Similarly, topical treatment of *Bacillus licheniformis* FGE4 to larvae decreased consumption index, relative growth rate, conversion efficiency of ingested food, and digested food values. **Conclusion**: The presence of gut bacteria with high chitinase activity negatively affects insect health. Utilizing gut-derived bacterial isolates with specific insecticidal traits offers a promising avenue to control fall armyworms. This research suggests a potential strategy for future pest management.

Keywords: fall armyworm; gut-associated bacteria; chitinase producing gut-associated bacteria; quantitative food use efficiency

1. Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is an important pest to numerous crops, including maize (*Zea mays*), cotton (*Gossypium hirsutum*), and sorghum (*Sorghum bicolor*). The production of maize in India rose from 29 million tonnes in 2018–2019 to 35.91 million tonnes in 2022–2023. Due to its industrial usage, maize is a low-input and high-profit crop. However, the invasive FAW, *Spodoptera frugiperda*, represents a new threat to maize cultivation [1]. Annual output losses from FAW ranged from 8.3 million to 20.6 million tons [2]. Chemical insecticides are frequently used to manage FAW in maize, which has the potential to build resistance over many generations and negatively impact natural enemies [3]. FAW has been demonstrated to be susceptible to entomopathogens such as nuclear polyhedroviruses (SfMNPV), *Metarrhizium rileyi*, and *Nomuraea rileyii* and were reported to cause larval infection and mortality [4–6].

To combat entomopathogens, insects produce reactive oxygen species (ROS), gut phagocytosis, antimicrobial proteins (AMPs), and phenoloxidase (PO) [7]. The immune systems of insects make it difficult for entomopathogens to survive and function effectively in the insect's gut [8]. Mumcuoglu *et al*. [9] revealed that the indigenous gut bacteria could be used for control measures because the exogenous bacteria fed to the insects were killed during the passage through the gut. The indigenous microbiota may quickly adjust to changes in the intestinal environment [10]. Hence, insect gut bacteria would be the better way to implement insect pest management.

Interestingly, insect gut microorganisms are crucial to their biology because they establish symbiotic relationships and cause host insect disease [11]. Zhang *et al*. [12] reported that *Enterobacter hormaechei* promoted housefly larvae growth by inhibiting harmful *Pseudomonas aeruginosa*, *Providencia stuartii*, and *Providencia vermicola* and improved the reproduction of beneficial bacteria. *Enterococcus*, *Comamonas*, and *Elizabethkingia* were reported to be responsible for most functional alterations in *S. frugiperda* microbiota [13]. Rozadilla *et al*. [14] described that archaea and bacteria from the *S. frugiperda* gut play a significant role in the nutritional requirement of fifth-instar larvae. The gut microorganisms of *S. frugiperda* fluctuate throughout developmental stages and show vertical transmission of bacteria, while nutrition and the environment

might influence gut bacteria [15]. Gut bacteria produce iron-chelating compounds termed siderophores to collect iron from the host insect for bacterial development and proliferation [16]. These siderophores protect the host insects from entomopathogens [17]. According to Krishnamoorthy *et al*. [8], *Bacillus* sp. in papaya mealybugs helped detoxify profenophos and chlorpyrifos pesticides used for mealybug management.

A protein–carbohydrate matrix with a chitin concentration ranging from 3 to 13% v/v forms the insect's peritrophic membrane [18] and is crucial for the insect's food uptake, growth, and development. The development of insects is influenced by the changes caused by the peritrophic membrane's chitin and protein compositions [19]. Since it is difficult for insects to develop resistance to microbial enzymes, an environmentally benign tactic is to use gut bacterial enzymes to break down the insect's structural component by taking advantage of its chitinous morphological structure [20]. Chitinases produced by gut bacteria are employed to degrade the insect's cuticle partially and they can reduce insect growth by decreasing feeding rate and body weight, leading to insect mortality [21,22]. It was found that the chitinase-producing *Serratia marcescens* caused the highest mortality in the treated larvae of *Spodoptera littura* and was suggested as a biocontrol agent against *Spodoptera littura* [23]. Harrison *et al*. [24] demonstrated that the protease enzyme can act as insecticidal when overexpressed. The protease toxic activity can occur in various areas of the insect body, such as the midgut, hemocoel, and cuticle. The present study aimed to isolate and select gut-associated FAW bacteria based on their chitinase and protease activities. In addition, attempts were made to control FAW *in vitro* by providing chitinase-producing bacteria in the diet and topical application in crude enzyme form and cell suspension. We also examined the harmful effects on the growth and development of their insect hosts.

2. Materials and Methods

2.1 Insect Collection

FAW used in this study was obtained from laboratory-grown and infected maize field populations. Nearly forty larvae were collected. The larval collection was performed between November 2021 and January 2022 in the maize fields, which had not been exposed to any of the pesticides at Tamil Nadu Agricultural University, Coimbatore, India (11.0123° N, 76.9355° E) and Dharapuram (10.7343° N, 77.51861° E), Tamil Nadu, India. The laboratory-reared FAW populations were obtained from the Department of Plant Biotechnology and Molecular Biology, Tamil Nadu Agricultural University, Coimbatore, India. The larvae were raised in a laboratory for nearly 190 generations using an artificial feed prescribed by "CIMMYT" [25] at 25 $\pm$ 1 °C, 16:8 h light/dark photoperiod, and 75% relative humidity.

2.2 Isolation of Cultivable Gut Bacteria Associated with S. frugiperda

Since fourth (IV) and fifth (V) instar larvae inflict substantial damage upon their hosts, these instar larvae were selected to isolate gut-associated bacteria. To remove the transitory microbiomes, 25 larval instars were selected from the laboratory, reared and field-caught populations, and left to fast for a whole day. Subsequently, the larvae were surface disinfected for five minutes using 70% ethanol and washed three to five times with sterile distilled water [26]. Under sterile conditions, the larvae were dissected, and gut samples were collected in 0.1 M phosphate buffer (pH 7.0). The gut samples were homogenized in a sterile pestle and mortar, serially diluted, and spread in eleven different growth media-containing Petri plates. The used growth media were Corn Meal agar, Czapek Dox agar, De Man, Rogosa, and Sharpe (MRS) agar, Eosin-methylene blue (EMB) agar, Endo agar, Luria–Bertani agar, MacConkey agar, nutrient agar, Reasoner's 2A (R2A) agar, Tryptose soy agar, and yeast extract peptone dextrose (YPD) agar (HiMedia, Mumbai, India). The plates containing the gut suspensions were incubated for 72 hours at 28 $\pm$ 2 °C and checked every 24 hours for the formation of new colonies. Colonies were differentiated into singular morphotypes based on size, color, and shape. Each morphotype was represented by a single isolate on new plates. After five or six streaks, the purity of the culture was determined using a light microscope (Magnus MLX, New Delhi, India). Purified bacterial isolates were stored in 50% glycerol at –80 °C.

2.3 Functional Significance of Gut Bacteria Associated with S. frugiperda

2.3.1 Detection of Chitinase Activity

Chitin degradation was quantified using the 3,5-dinitrosalicylic acid (DNS) assay. Briefly, bacterial isolates (1×10^7 CFUs/mL) were inoculated (1% v/v) into 25 mL of nutrient broth (Himedia) supplemented with 3% colloidal chitin and incubated at 28 $\pm$ 2 °C and 200 rpm/min for 48 h. After collecting the bacterial cells by centrifugation at 10,000 rpm for 20 minutes at 4 °C, the supernatant containing the crude enzyme was collected in a microcentrifuge tube. The chitinolytic activity was quantitatively assessed in a spectrophotometer (Shimadzu, Kyoto, Japan) by adding 0.1 M McIlvaine buffer (pH 6.0) and 1.5 mM potassium ferricyanide solution to the crude enzyme solution. After measuring the absorbance at 420 nm, the enzyme activity was calculated using N-acetylglucosamine (NAG) concentration as a standard. One unit of enzyme activity was defined as the amount of enzyme releasing one µmol of NAG per minute per mL [27].

2.3.2 Detection of Protease Activity

Skim milk agar plates were used to assess the protease enzyme activity of FAW gut-associated bacterial isolates qualitatively [28]. The bacterial isolates (1×10^7

CFUs/mL, 10 µL) were spotted on skim milk agar plates and incubated at 28 ± 2 °C for 48 h. The halo zone surrounding the spots is a visual cue that the bacteria can produce protease. The diameter of the halo zone surrounding the spotted cultures was assessed to ascertain the protease activity. The protease activity was described as protease (%) = diameter of the halo zone/diameter of the colony × 100.

2.3.3 Detection of Siderophore Production

Siderophore-producing bacterial isolates were qualitatively assessed using Chrom Azurol S (CAS) agar plates, as described by Dutta *et al.* [29]. The succinate medium was prepared separately (pH 6.5), then CAS indicator solution was added to the medium, adjusted to pH 7.0, and autoclaved. Next, the bacterial cultures (1×10^7 CFUs/mL, 10 µL) were spotted on the medium and were incubated at 28 ± 2 °C for 48 h. While the medium is blue, the presence of an orange halo zone around the spotted culture indicates the production of siderophore by the bacterium. The siderophore production by the bacterial isolates was calculated as siderophore production (%) = diameter of the halo zone/diameter of the colony × 100.

2.4 Molecular Characterisation of the Gut Bacterial Isolates

For molecular identification, the genomic DNA of bacterial isolates with high chitinase and protease activity was extracted using the CTAB method. The 16S rRNA gene was amplified from the isolated DNA using the universal primers 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492 R (5′-GGTTACCTTGTTACTT-3′). A total of 30 µL of the reaction mixture was used with the following conditions for PCR cycling: (1) at 95 °C for 5 min; (2) 30 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; (3) 72 °C for 10 mins. The DNA concentration measured on a Nanodrop at 260/280 nm was 1.8 to 2, and the DNA quality was visualized using horizontal gel electrophoresis. DNA sequencing for the isolates (from FGE1 to FGE21) was conducted using an ABI 3730xl (48 capillary) instrument (ThermoFisher, Waltham, MA, USA) in Bioserve Pvt. Ltd., Hyderabad, India. The acquired nucleotide sequences were uploaded to the NCBI database, and GenBank accession numbers were obtained.

2.5 Nutritional Significance of Gut Bacteria Associated with S. frugiperda

Nitrogen-fixing ability, zinc, silica, and phosphate solubilization efficiency were assessed to identify the role of gut-associated bacteria in host insect nutrition. The nitrogen-fixing ability of gut bacterial isolates was assessed by growing the isolates in a nitrogen-free bromothymol blue malic acid medium (Nfb). The color change of the medium from green to blue indicated that the isolates could fix nitrogen [8]. The zinc and silica solubilization efficiency was detected by spotting the isolates (10 µL, 1×10^7 CFUs/mL) on Bunt and Rovira medium supplemented with 0.1% zinc carbonate and magnesium trisilicate, respectively. The phosphate solubilization efficiency was detected by spotting the isolates on Sperber's hydroxy apatite medium. A halo zone around the colonies indicated the solubilization efficiency by bacterial isolate, and the solubilization efficiency (SE) was calculated as described previously [30].

2.6 Effect of Chitinase on Quantitative Food Use Efficiency

The efficient chitinase-producing *Bacillus licheniformis* FGE4 and *Enterobacter cloacae* FGE18 isolates from the gut of FAW were chosen to test their effect on the nutritional indices of host insects as a consequence of chitinase. Hence, both isolates were added as food supplements and topically applied to the larvae. To extract the crude chitinase enzyme, the bacterial isolates were cultured in a liquid nutrient medium supplemented with 0.3% colloidal chitin. The cells were separated by centrifugation (10,000 rpm at 4 °C for 5 min), and the enzyme-containing supernatant was then purified with a membrane filter before being utilized in the bioassay. The cell suspension was prepared by washing the pellets in 0.05 mol phosphate buffer. The phosphate buffer was prepared by mixing sodium phosphate dibasic stock (0.5 M) and sodium phosphate monobasic stock (0.5 M), and the pH was adjusted to 7.0. After being washed twice with the buffer, the cell pellets were suspended in sterilized distilled water and were used for experiments. The following were the test solutions (treatments) used per 2 grams of CMMYT diet for conducting bioassay: (i) 1 mL of the cell suspension (10^7 CFUs/mL) + 1 mL of 0.05 mol phosphate buffer (pH 7.0), (ii) 1 mL of the cell suspension + 2 mL of the filtered sterilized crude enzyme, (iii) 2 mL of the crude enzyme, and (iv) 2 mL of the phosphate buffer (pH 7.0). The same treatment structure was also adopted for the topical application of test solutions on the larvae. The test solutions were sprayed on each larva at a 0.5 mL/larva rate. Ten larvae were maintained for each treatment, and three replications were maintained for each treatment. The experiment lasted three days, with an observation for every 24 hours. The consumption, growth rate, and post-ingestive food use efficiencies, including the consumption index (CI) = E/TA, relative growth rate (RGR) = P/TA, approximate digestibility (AD) = 100(E-F)/E, the efficiency of the conversion of the ingested food (ECI) = 100 P/E, the efficiency of the conversion of the digested food (ECD) = 100 P/(E-F), were calculated gravimetrically on a dry weight basis [31], where A = the mean dry weight of the larvae during the experimental period (T), E = the dry weight of the food eaten, F = the dry weight of the feces produced, and P = the dry weight gain of the larvae.

2.7 Statistical Analysis

ANOVA was used to analyze the data, and the General Linear Models Tukey's HSD test was used to compare the

Table 1. Functional significance of FAW gut-associated bacteria.

Isolates	Closest match	Similarity %	Length (bp)	NCBI accession number	Chitinase activity (µmol/min/mL)	Protease activity (%)	Siderophore production (%)
FGE1	*Bacillus amyloliquefaciens* longA	98.65	1505	OP070959	1.78 ± 0.02^{c}	ND	ND
FGE2	*Enterobacter cloacae* TBMAX89	99.59	1472	OP068371	1.72 ± 0.02^{c}	34.62 ± 0.05^{h}	50 ± 1.64^{a}
FGE3	*Klebsiella variicola*AHKv-S01	97.54	1450	OP070061	1.54 ± 0.01^{d}	ND	78.57 ± 0.32^{c}
FGE4	*Bacillus licheniformis* DS3	98.11	1457	OP070050	2.1 ± 0.07^{b}	ND	ND
FGE5	*Bacillus subtilis* ANA4	98.75	1476	OP070059	1.04 ± 0.01^{f}	75 ± 0.59^{b}	66.67 ± 0.94^{e}
FGE6	*Enterococcus mundtii* UDFX4	98.62	1464	OP081022	0.94 ± 0.03^{g}	28 ± 0.61^{i}	ND
FGE7	*Kocuria turfanensis* NL52	98.78	1439	OP070058	0.68 ± 0.01^{h}	27.27 ± 0.88^{i}	ND
FGE8	*Bacillus subtilis* OH2377A	95.47	1465	OP070955	0.16 ± 0.00^{j}	65.38 ± 0.07^{c}	ND
FGE9	*Enterococcus mundtii*15-1A	98.38	1487	OP081005	0.22 ± 0.01^{j}	51.85 ± 1.05^{f}	20 ± 1.02^{b}
FGE10	*Bacillus thuringiensis* SRG2	95.13	1452	OP070946	0.22 ± 0.03^{j}	28.57 ± 0.28^{i}	ND
FGE11	*Enterococcus* sp. RJ-7	98.9	1500	OP070961	1.08 ± 0.04^{f}	ND	ND
FGE12	*Pantoea agglomerans* P18	97.66	1469	OP070952	ND	87.5 ± 0.18^{a}	ND
FGE13	*Enterobacter hormaechei* DS02Eh01	99.25	1441	OP070948	ND	39.29 ± 0.67^{g}	ND
FGE14	*Enterococcus durans* 4434	98.82	1459	OP070943	ND	16.67 ± 1.28^{j}	ND
FGE15	*Enterobacter mori* YIM Hb-3	98.19	1503	OP070944	0.14 ± 0.01^{j}	9.52 ± 0.65^{k}	33.33 ± 0.87^{d}
FGE16	*Enterobacter asburiae* A2563	99.14	1426	OP070960	1.42 ± 0.10^{e}	ND	ND
FGE 17	*Bacillus cereus* P3B	97.78	1525	OP070957	0.68 ± 0.03^{b}	26.06 ± 0.29^{i}	ND
FGE 18	*Enterobacter cloacae* R6-366	98.36	1461	OP070949	2.3 ± 0.03^{a}	51.85 ± 0.94^{f}	ND
FGE19	*Bacillus halotolerans* BBRIST011	96.00	1462	OP070947	0.4 ± 0.00^{i}	61.54 ± 2.43^{d}	ND
FGE20	*Bacillus velezensis* JF37	96.03	1475	OP070950	0.94 ± 0.24^{g}	60 ± 0.56^{d}	ND
FGE21	*Bacillus pumilus* SBMP2	98.48	1456	OP070926	ND	54.55 ± 1.14^{e}	ND

The first column in the table represents the gut bacterial isolates associated with fall armyworm (FAW). FGE1–FGE3 represents isolates from the fourth instar field-caught FAW population, FGE4–FGE12 represents isolates from the fifth instar artificial-diet-reared FAW population, FGE13–FGE14 represent isolates from the fifth instar field-caught FAW population, FGE15–FGE21 represents isolates from fourth instar artificial-diet-reared FAW population. Values in each column are the mean of three replications ± standard error (SE). The means in the columns with letters a–k are significantly different at 0.05 levels (Tukey's HSD test). ND, not detected.

means. The square root and arcsine transformations were used for the data transformation of numbers and percentages. IBM SPSS (SPSS, 2013, IBM Corp., Armonk, NY, USA) was used for all data analysis.

3. Results

3.1 Gut Bacterial Isolation

A total of 111 morphologically distinct bacteria were isolated from the IV and V instars of the field-caught and laboratory-reared (with artificial diet) FAW populations. Of the 111 isolates, 38 and 29 were IV and V instars, respectively, from the laboratory-reared FAW population; a further 20 and 24 isolates were recovered from IV and V instar larvae, respectively, from the field-caught FAW population. No bacterial colonies were detected in the gut suspensions from the IV and V instar artificial diet-reared larval populations or V instar field-caught FAW populations in the Corn Meal agar plates. In the MRS medium, bacterial colonies were only visible 48 hours after incubation. The maximum numbers of bacteria were found in the gut suspension of IV instar field-caught larvae from the NA medium. In contrast, the lowest numbers were found in the IV instar artificial diet-reared larvae from the Endo agar medium. Out of all the isolates from the gut samples of the larvae raised on an artificial diet, the TSA medium revealed the largest bacterial population. In contrast, the Endo agar medium revealed the lowest population.

3.2 Molecular Characterization

The 16S rRNA gene analysis revealed that the isolates recovered from *S. frugiperda* belonging to Firmicutes (also known as Bacillota) contain three different genera, with *Bacillus* being the predominant one. Additionally, our results reported three different genera of Gammaproteobacteria. The nucleotide sequences of the recovered bacterial isolates were subjected to homology searches in DNA databases, which revealed that the sequences of the FGE1, FGE4, FGE5, FGE8, FGE10, FGE17, FGE19, FGE20, FGE21 isolates showed a 95% to 98% similarity with the 16S rRNA gene sequences for the *Bacillus* species and FGE2, FGE13, FGE15, FGE16, and FGE18 (99%) were homologous with the *Enterobacter* species. Similarly, FGE6, FGE9, FGE11, and FGE14 showed 98% similarity with the *Enterococcus* species (Table 1).

3.3 Functional Significance of Cultivable Bacteria Isolated from S. frugiperda

Among 111 bacterial isolates, only 81 were chitinase positive, and 37 were protease positive (Table 1). The maximum chitinolytic activity (2.3 ± 0.03 µmol of N-acetyl glucosamine/min/mL was observed in *Enterobacter cloacae* FGE18 from the IV instar FAW reared on an artificial diet followed by *Bacillus licheniformis* FGE4 (2.1 ± 0.07 µmol/min/mL) from V instar reared in the same conditions. In contrast, the lowest activity was recorded in *Enterococcus muntii* FGE9 and *Bacillus thuringiensis* FGE10 (0.22 ± 0.01 µmol of N-acetyl glucosamine/min/mL and 0.22 ± 0.03 µmol of N-acetyl glucosamine/min/mL, respectively) from V instar reared on an artificial diet. Among 37 protease-positive isolates, *Pantoea agglomerans* FGE12 from V instar artificially reared larvae showed maximum protease activity (87.5 ± 0.18%) followed by *Bacillus subtilis* FGE5 (75 ± 0.59%). *Enterobacter mori* FGE15 from the IV instar artificially reared population showed minimum protease activity of 9.52 ± 0.65%. Among the 111 bacterial isolates, only 21 were screened based on their chitinase and protease activities with different morphotypes. Further, these 21 isolates were characterized for their siderophore production, of which only five isolates showed siderophore positive. *Klebsiella variicola* FGE3 from the IV instar field-caught population showed the highest siderophore production (8.5 ± 0.32%), while the lowest value was noted in *Enterococcus muntii* FGE9 (20 ± 1.02%) from the V instar larvae reared on an artificial diet.

3.4 Nutritional Significance of Gut Bacteria Associated with S. frugiperda

Among the 21 screened gut bacterial isolates, 9 isolates (*Klebsiella variicola* FGE3, *Bacillus subtilis* FGE5, *Bacillus subtilis* FGE8, *Pantoea agglomerans* FGE12, *Enterobacter hormaechei* FGE13, *Enterobacter asburiae* FGE16, *Bacillus cereus* FGE17, *Enterobacter cloacae* FGE18, *Bacillus pumilus* FGE21) were found to fix nitrogen in the medium (Table 2). Eleven isolates were found to solubilize zinc, with the maximum solubilization shown by *Enterobacter hormaechei* FGE13 (81.82 ± 2.43%). Among the seven phosphate solubilizing bacteria, *Enterobacter hormaechei* FGE13 (87.5 ± 4.31%) exhibited the maximum solubilization efficiency. In addition, the silica solubilization efficiency was noted in 10 bacterial isolates, with *Bacillus cereus* FGE17 (92.5 ± 3.66%) showing the highest silica solubilization efficiency.

3.5 Effect of Chitinase on Quantitative Food Use Efficiency

There was a decrease in consumption rate (1.87 ± 0.45 mg (ingested food)/mg (average larval weight)/day) and relative growth rate (0.43 ± 0.01 mg (ingested food)/mg (average larval weight)/day) of larvae treated with crude chitinase of *Bacillus licheniformis* FGE4 through topical application than the control (4.35 ± 0.23 and 0.18 ± 0.01 mg (ingested food)/mg (average larval weight)/day) (Fig. 1). Similarly, there was a 95.89% reduction in the consumption index and a 23.68% reduction in the growth of the larvae treated with the crude chitinase of *Enterobacter cloacae* FGE18 through topical application than the control larvae. There was a 52.63% growth reduction in the larvae treated with the crude chitinase of *Enterobacter cloacae* FGE18 through the feed (Fig. 2). Additionally, the ECI and ECD values were reduced compared to the control in a diet amended with crude chitinase of *Enterobacter cloacae* FGE18, and there were 55.16% and 60.15% reductions in both ECI and ECD values, respectively. The percentage reduction in ECI and ECD values were 40.23 and 55.96, respectively, in the case of larvae treated with the crude chitinase of *Enterobacter cloacae* FGE18 through topical application. The consumption index and efficiency of conversion of ingested (ECI) food values were found to be less (1.87 ± 0.45 mg (ingested food)/mg (average larval weight)/day and 14.24 ± 0.69%) when the larvae were topically treated with the cell pellets of *Bacillus licheniformis* FGE4. Similar results were found, such as a decreased rate in the consumption index and ECI values in cell pellets of *Enterobacter cloacae* FGE18 when the larvae were treated topically.

4. Discussion

Understanding the contribution of gut bacteria to host insect activities, such as antagonistic against invading pathogens, detoxification of pesticides, and host feeding, requires determining the species of bacteria and their probable involvement in the host insect gut environment. This study revealed the presence of different cultivable FAW gut bacteria, which were collected from field conditions and laboratory-reared populations. Based on the molecular characterization, the cultivable gut bacterial isolates belong to 18 different bacterial species, *viz., Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus subtilis*, *Klebsiella variicola*, *Kocuria turfanensis*, *Enterococcus mundtii*, *Enterococcus durans*, *Bacillus thuringiensis*, *Enterococcus* sp., *Pantoea agglomerans*, *Enterobacter hormaechei*, *Enterobacter mori*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Bacillus cereus*, *Bacillus halotolerans*, *Bacillus velezensis*, and *Bacillus pumilus*. Similar reports were found in *S. frugiperda*-infested maize fields in Shaanxi Province, where *Enterococcus* and *Enterobacteriaceae* members, such as *Enterobacter*, *Klebsiella*, *Pantoea*, *Escherichia*, *Rhodococcus*, and *Ralstonia*, predominated in the guts of both adults and larval stages [15]. The genus *Enterococcus* was the most common in the FAW gut samples obtained from eastern parts of India, followed by *Klebsiella* sp. and *Enterobacter* sp., with a small proportion of *Raoultella*, *Citrobacter*, *Leclercia*, and *Pantoea* [32]. Acevedo *et al.* [33] also reported that *S. frugiperda* oral secretions contained *Pantoea*, *Enterobacter*, *Raoultella,* and *Klebsiella*. Indiragandhi *et al.* [17] reported the different bacterial phylotypes present in the insecticide-resistant, susceptible,

Table 2. Nutritional significance of FAW gut-associated bacteria.

Isolates	Nitrogen fixation	Solubilization (%)		
		Zinc	Silica	Phosphate
Bacillus amyloliquefaciens FGE1	-	ND	ND	ND
Enterobacter cloacae FGE2	-	ND	ND	ND
Klebsiella variicola FGE3	+	7.15 ± 0.31^{b}	40 ± 1.09^{b}	33.34 ± 0.16^{c}
Bacillus licheniformis FGE4	-	27.28 ± 0.27^{d}	ND	ND
Bacillus subtilis FGE5	+	ND	ND	ND
Enterococcus mundtii FGE6	-	33.34 ± 1.27^{b}	ND	ND
Kocuria turfanensis FGE7	-	ND	20 ± 0.7^{c}	ND
Bacillus subtilis FGE8	+	ND	ND	ND
Enterococcus mundtii FGE9	-	20 ± 0.75^{c}	ND	ND
Bacillus thuringiensis FGE10	-	ND	ND	ND
Enterococcus sp. FGE11	-	ND	ND	ND
Pantoea agglomerans FGE12	+	10 ± 0.13^{e}	50 ± 2.24^{a}	66.67 ± 1.67^{c}
Enterobacter hormaechei FGE13	+	81.82 ± 2.43^{a}	81.82 ± 2.45^{b}	87.5 ± 4.31^{a}
Enterococcus durans FGE14	-	44.45 ± 0.45^{i}	10 ± 0.14^{b}	ND
Enterobacter mori FGE15	-	ND	ND	ND
Enterobacter asburiae FGE16	+	55.56 ± 2.43^{h}	53.85 ± 1.23^{e}	50 ± 2.56^{b}
Bacillus cereus FGE17	+	66.67 ± 1.65^{f}	92.5 ± 3.66^{f}	83.34 ± 2.28^{a}
Enterobacter cloacae FGE18	+	50 ± 1.81^{i}	83.34 ± 0.39^{d}	66.67 ± 2.75^{d}
Bacillus halotolerans FGE19	-	ND	23.08 ± 0.11^{e}	ND
Bacillus velezensis FGE20	-	ND	ND	ND
Bacillus pumilus FGE21	+	62.5 ± 1.56^{g}	81.82 ± 2.45^{c}	50 ± 2.25^{b}

The first column in the table represents the gut bacterial isolates associated with fall armyworm (FAW). FGE1–FGE3 represents isolates from the fourth instar field–caught FAW population, FGE4–FGE12 represents isolates from the fifth instar artificial-diet-reared FAW population, FGE13–FGE14 represents isolates from the fifth instar field-caught FAW population, FGE15–FGE21 represents isolates from the fourth instar artificial-diet-reared FAW population. Values in each column represent the mean of three replications $\pm$ standard error (SE). The means in the columns with letters a–i are significantly different at 0.05 levels (Tukey's HSD test). +, positive result; -, negative result; ND, not detected.

field-caught population of *Plutella xylostella*. The absence of specific bacteria in the larval gut limits its pupation and successful adult emergence [34]. Hence, the cultivable bacteria isolated from the FAW might have a significant role in the biology of *S. frugiperda* larvae and even with adult development. The study conducted by Sivakumar *et al*. [35] reported that the gut bacterium *B. pumilus* associated with *A. biguttula biguttula* has a role in host insect nutrition and defense for the first time.

Chen *et al*. [36] suggested that *Enterococcus* may affect the metabolism level in the gut of *S. frugiperda* by aiding carbohydrate transport and energy production. *Enterococcus* spp., identified in this study, may contribute to the defense response to FAW. Research has demonstrated that members of *Enterobacteriaceae* are involved in the metabolism of sugar in larvae and digestion, defense, courtship, and reproduction [37]. In the present study, cultivable *Enterobacteriaceae* members were also isolated. The highest bacterial population was found in the NA medium and the lowest in the MacConkey agar medium among the field-caught FAW larval gut samples of IV and V instar larvae. Similar results were observed in the cultivable gut bacteria isolated from the diamondback moth, *Plutella xylostella,* where the highest bacterial population was observed in NA medium, and the lowest number of bacterial populations was observed in MacConkey agar [17]. The gut of the field-caught population may have the highest bacterial population level because of the increased nutritional availability from the natural host plants; however, the artificially reared population harbored a lower abundance of the bacterial population. The increased number of Firmicutes in the guts of *S. frugiperda* larvae might result from the larvae's improved ability to absorb various nutrients [37].

The exoskeleton and peritrophic membrane of insects serve as a physicochemical defense and are composite materials predominantly composed of chitin and protein, with the latter also including trace amounts of lipids, catecholamine metabolites, minerals, and other minor constituents [38]. The pathogens or pests may be exposed to chitinases at unsuitable concentrations or stages of development to make them more susceptible to host defenses [38]. Consequently, bacterial isolates in the current study were screened based on their chitinolytic and proteolytic activity. Of the 111 gut bacterial isolates, 81 could produce chitinase, 37 were protease producers, and 5 were siderophore producers. The gut flora controls the thickness

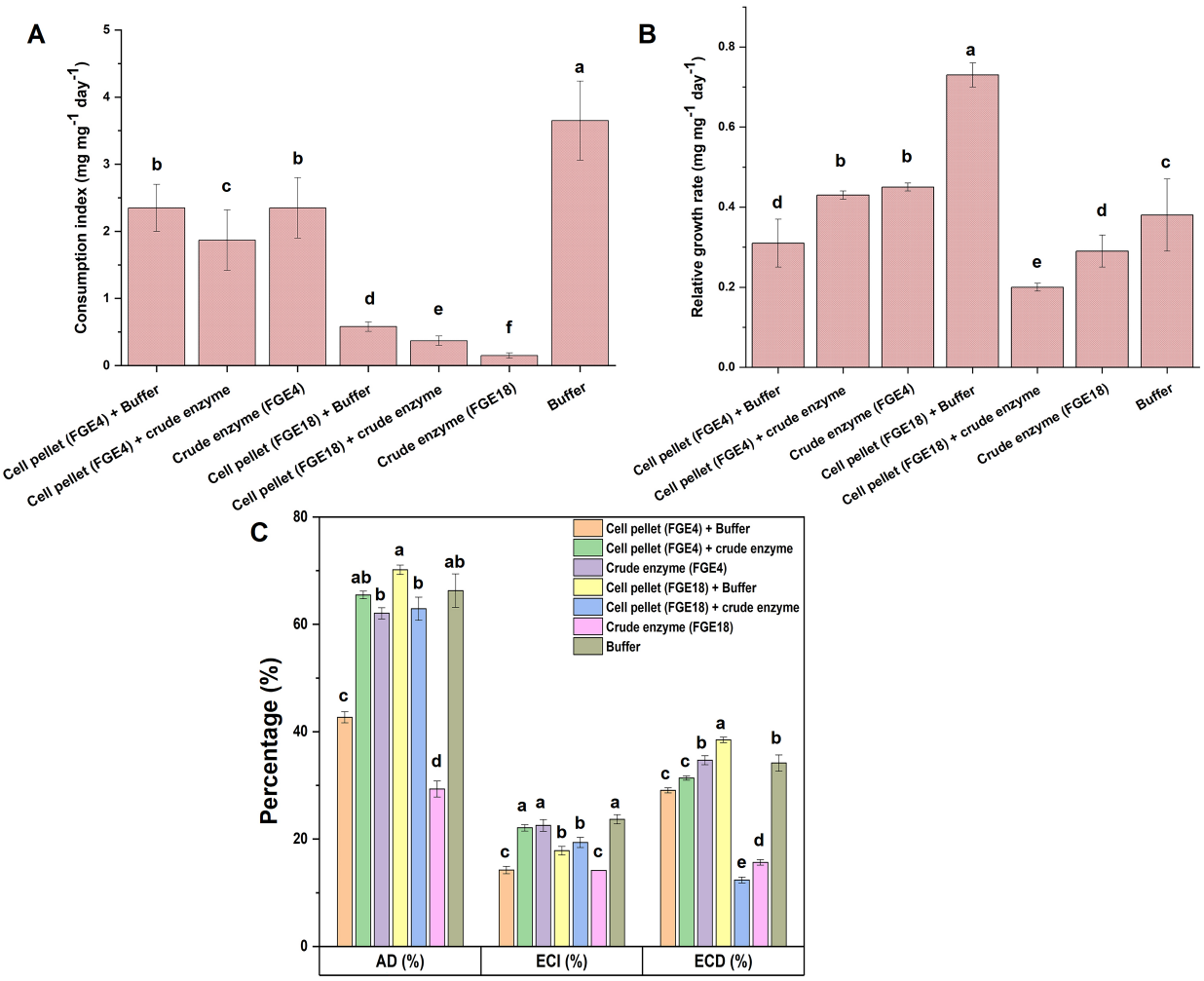

Fig. 1. Effect of chitinase on quantitative food use efficiency through topical application. Each panel represents a mean of three replicates, the error bar indicates standard error, and panels with the letter(s) a–f are significantly different at 0.05 levels (Tukey's HSD test). (A) Consumption index, (B) relative growth rate, (C) approximate digestibility (AD), efficiency of conversion of ingested food (ECI), and efficiency of conversion of digested food (ECD).

of the peritrophic membrane, which affects how nutrients pass through the insect gut [39]. Chitinase and protease-producing gut bacteria were found to enter the host gut through feeding and disturb the thickness of the peritrophic membrane, which led to a nutritional imbalance in the host insect and mortality [8,22]. Similarly, the protease enzymes produced by *Xenorhabdus nematophila* could suppress the insect's immune system [40]. Due to the low iron concentration in the gut environment, siderophore production is believed to be widespread in insect gut bacteria. The siderophores are produced and secreted by bacteria complexes with iron outside the bacterial cell, which they then deliver to the insect [41]. Some opportunistic pathogens that require iron for pathogenicity are found in the gut of insects. By generating siderophores, they chelate the iron from the consumed insect feed. As a result, an insect may die from iron toxicosis if it consumes too much iron [42].

Insect gut bacteria correlate with their insect partners in terms of nutrition [43]. The droplets of poplar and willow borer, *Cryptorhynchus lapathi*, contained bacterial enzymes involved in nitrogen and sulfur metabolism and were also involved in the biosynthesis of essential amino acids and vitamins [7]. The bacteria isolated from the gut samples in the present study showed nitrogen fixation, zinc, silica, and phosphate solubilization. The organisms involved in nutrient provisioning and insect physiology can be altered or eliminated to retard insect growth, which can be useful for pest control. Eliminating gut bacteria with antibiotics impaired the growth and development of adult host insects [34].

In the present study, the extracted crude chitinase enzyme and chitinase-producing bacteria were supplemented in their diet, fed to the larvae, and topically sprayed on the larvae. There was a 98.63% reduction in the consumption index of larvae treated with the crude chitinase enzyme plus chitinase-producing *E. cloacae* FGE18 through topical application, and the growth reduction was also observed in both feeds of supplemented and topically infected lar-

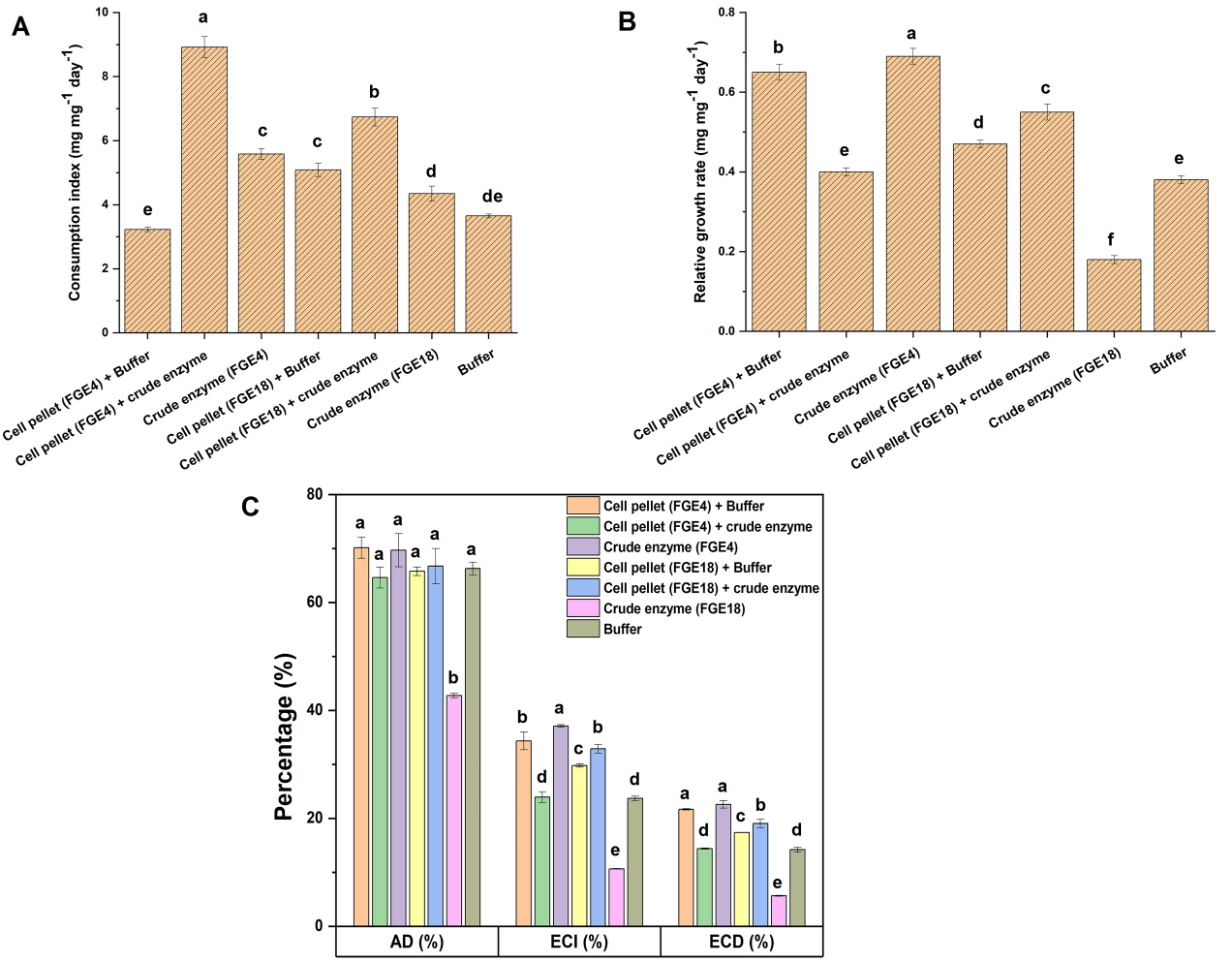

Fig. 2. Effect of chitinase on quantitative food use efficiency through feed supplementation. Each panel represents mean of three replicates, the error bar indicates standard error, and panels with the letter(s) a–f are significantly different at 0.05 levels (Tukey's HSD test). (A) Consumption index, (B) relative growth rate, (C) approximate digestibility (AD), efficiency of conversion of ingested food (ECI), and efficiency of conversion of digested food (ECD).

vae (52.63% and 23.68% growth reduction, respectively). The genomic sequence of *E. cloacae* subsp. *cloacae* indicated the presence of four chitinases and two N-acetylglucosaminidases that may be involved in chitin breakdown. Furthermore, the *E. cloacae* genome contains genes that code for one CBM-33 lytic polysaccharide monooxygenase and one polysaccharide deacetylase, which may play an important role in the depolymerization of chitin [44]. Additionally, Liao *et al.* [45] claimed the role of *E. cloacae* insecticidal protein in killing the host insect, *Galleria mellonella* larvae, by destroying or inhibiting their host immune response. In the case of *Bacillus licheniformis* FGE4, decreased ECI and ECD values were found in the topically applied larval population. These results were similar to the experiments conducted using transgenic tobacco plants expressing the *Manduca sexta* chitinase gene to feed tobacco budworms [46], and the results showed a reduction in larval biomass and feeding damage. In our investigation, most larval parameters exhibited lower values when the larvae were treated topically with cell suspension and crude enzymes of both isolates. Similar results were obtained by Kim *et al.* [47], where they described that chitinolytic and proteolytic effects in the culture supernatant of *Beauveria bassiana* could cause death when topically sprayed against aphids (*Aphis gossypii*). Harrison and Bonning [24] demonstrated that cuticle-degrading proteases such as PR1A may be hazardous to insects when provided topically. The colonization of chitinase-producing bacteria and the production of chitinase in the gut may lead to damage in the peritrophic membrane of the insect gut and cause diffusion of nutrients, which were similar in the mode of action of permethrin insecticides and delta endotoxins of *Bacillus thuringiensis* [21]. In evidence of this, several researchers have demonstrated the insecticidal properties of the chitinases from diverse microorganisms. Additionally, chitinolytic bacteria that produce protease, siderophores, and secondary metabolites were discovered to be the best for controlling nematodes [48]. The growth of the to-

bacco caterpillar, *Spodoptera litura*, was hindered by purified chitinases from *B. subtilis*. A talc-based formulation of *Pseudomonas fluroescens* and chitin has been observed to decrease the incidence of the leaf folder *Cnaphlocrocis medinalis* in rice by 56.1% [49].

5. Conclusion

Our study concluded that gut-inhabiting bacteria with high chitinase activity can negatively influence the insect's health and survivability. Hence, enhancing them would be an alternate strategy in insect pest management. Chitinase-producing *Bacillus licheniformis* FGE4 and *Enterobacter cloacae* FGE18 isolated from the gut of *S. frugiperda* larvae can be used against the FAW larvae. However, the application of these identified bacteria in the field conditions, evaluating their persistence in the field, and method of applications for effective management of insect pests needs to be thoroughly studied in the future to drive this approach more effectively for the benefit of the farming community.

Abbreviations

FAW, Fall Armyworm; SfMNPV, *Spodoptera frugiperda* Multicapsid Nuclear polyhedrosis Virus; PO, Phenoloxidase; ROS, Reactive Oxygen Species; AMP, Antimicrobial proteins; CIMMYT, International Maize and Wheat Improvement centre; EMB agar, Eosin Methylene Blue agar; R2A agar, Reasoner's 2A agar; TSA, Typtose Soy Agar; NA, Nutrient Agar; YPD, Yeast Extract Peptone Dextrose agar; MRS agar, de Man Rogosa and Sharpe agar; cfu, Colony Forming Unit; CAS, Chrom Azurol S; CTAB, Cetyl Trimethyl Ammonium Bromide; PCR, Polymerase Chain Reaction; NCBI, National Center for Biotechnology Information; CI, consumption index; RGR, relative growth rate; AD, approximate digestibility; ECI, the efficiency of the conversion of the ingested food; ECD, the efficiency of the conversion of the digested food.

Availability of Data and Materials

The data utilized and/or examined in the present study can be obtained from the corresponding author upon a reasonable request.

Author Contributions

NOG and RA designed the research study. RA contributed to the conception of the present work. TDW performed the research, analyzed the data and wrote the manuscript. PI and VB provided oversight, literature search and direction for the drafting of the manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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