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Plant growth-promoting bacteria from dung of indigenous and exotic cow breeds and their effect on the growth of pea plant in sustainable agriculture

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Abstract

Aim In the present study, cow dung from indigenous and exotic breeds of cow were explored for its microbial population, plant growth-promoting (PGP) traits, enzyme activity, antagonistic activity, and pot experiment studies to evaluate the effect of bioinoculants under net house conditions.

Materials and methods Physicochemical properties of cow dung, qualitative and quantitative method of PGP characterization, agar diffusion method for antifungal activity, 16Sr RNA gene sequencing method for identification, and pot experiment studies were performed.

Results Nitrogen (N), phosphorus (P), potassium (K), electrical conductivity (EC), and pH were found maximum in indigenous *Himachali Pahari* non-lactating cow, whereas highest microbial count was found in *Himachali Pahari* lactating cow. Fourteen cow dung isolates from different breeds were found positive for all PGP, enzyme, and antifungal activities except hydrogen cyanide (HCN) production and were selected for further studies. Quantitative estimation showed isolate JD3 and JD4 isolated from Jersey non-lactating cow best in phosphate (P) solubilization (127.79 µg ml^{-1}) and siderophore production (98.42%), whereas PL2 isolated from *Himachali Pahari* lactating cow was found best in IAA production (80.03 µg ml^{-1}). Maximum antifungal activity was found in Jersey lactating and non-lactating cow dung isolates (JL1, JL2, JL4 and JD1), against all tested fungal phytopathogens. *Microbacterium thalassium* strain PL3 was first time reported from *Himachali Pahari* lactating cow. Pot experiment studies found that maximum plant height was recorded in *Himachali Pahari* lactating isolate (PL2) (14.76 cm), germination percent was recorded highest in Jersey lactating cow dung isolates (JL4, JL1) and control (91.77%), minimum days to 1st flowering were found in Jersey isolate (JL4 and JD1) (49.33 days) treatment, and pod number per plant was noted maximum in *Himachali Pahari* non-lactating (PD5) (3.33). Maximum chemical properties, viz., N (313.60 kg ha^{-1}), P (40.31 kg ha^{-1}), and microbial count (4.7×10^8 cfu g^{-1}) were found in *Himachali Pahari* lactating cow isolate (PL2) treatment, while K (253.74 kg ha^{-1}) was found to be maximum in *Himachali Pahari* non-lactating cow isolate (PD3) which was statistically significant than initial values of microbial count, N, P, and K.

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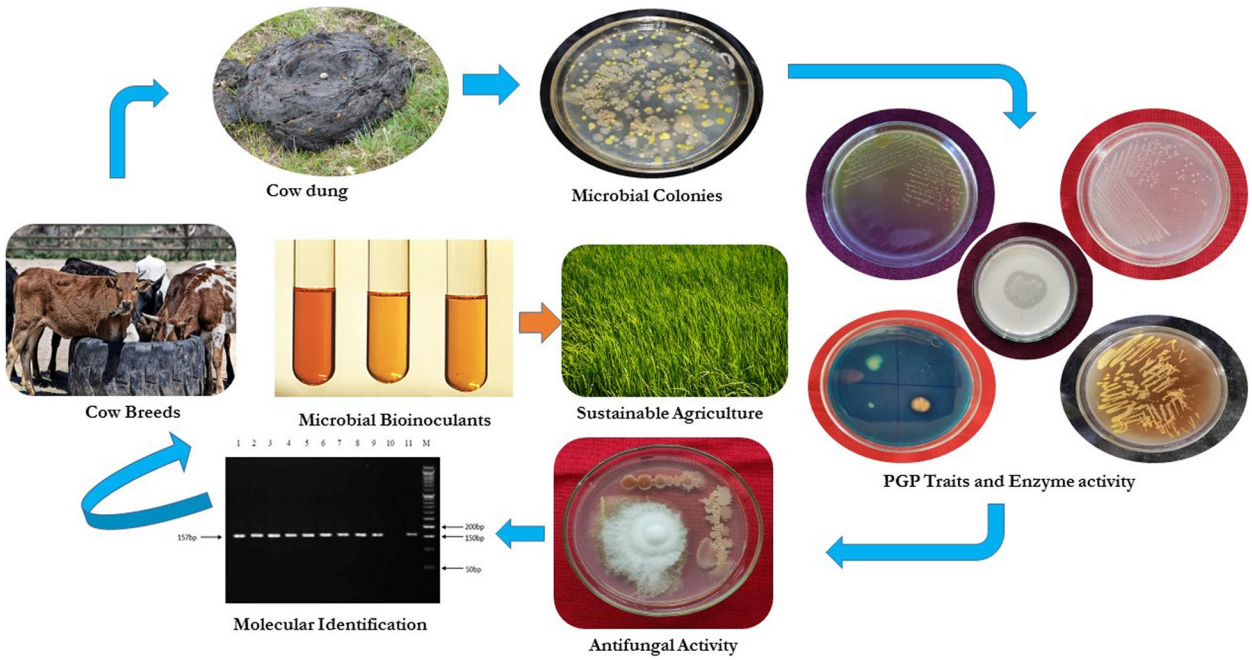
Impact statement These potential five strains, i.e., PL2, PD5, JL1, JL4, and JD1, isolated from cow dung of indigenous *Himachali Pahari* and exotic Jersey breeds irrespective of lactating and non-lactating in individual or in consortium can be potential means to protect, enrich, and flourish the soil microbial community as well as plant and soil health.

Keywords Antifungal activity, Enzyme activity, Plant growth-promoting traits, Indigenous cow, Exotic cow

Graphical Abstract



Introduction

In hilly and mountain regions of Himachal Pradesh (India), organic farming is practiced with negligible use of agrochemicals. Organic agriculture based on indigenous fermentation technologies like bio-formulations and liquid manures are used in organic farming. Cow dung is an excellent source of microorganisms and an integral component of these bio-formulations and liquid manures [1, 2]. The excreta while passing through the intestine washes off intestinal lining along with the microbes associated with it. These bio-formulations are rich in minerals and beneficial microorganisms, which promote the growth of plant, also offering eco-friendly and sustainable alternative soil inoculants and bio-pesticides [3, 4].

Since the ancient time, cow dung has been associated with agriculture and been incorporated in agricultural lands in order to bring about the nutritive value of crops and maintaining crop health. It contains variety of nutrients like nitrogen, phosphorus, potassium, magnesium, and calcium. Cow dung is the excreta of bovine animal with a 3:1 mixture of feces and urine containing essential nutrients [2]. The gut microbiome of bovine animal contains various microorganisms such as *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium*, *Bacillus subtilis*, and *Saccharomyces cerevisiae.* Cow dung is a mixture of bacteria and fungi, and as the cow dung grows older, the microbial community is altered with the soil contaminants and other bacteria not previously being a part of gut microbiota [5]. In addition to these nutrients and bacteria, cow dung contains beneficial plant growth-promoting bacteria (PGPB) responsible for further enhancement of plants by variety of direct and indirect mechanisms [6]. Cow dung is a rich source of PGPB that reside in the rhizosphere or the region surrounding the plant roots help in stimulating plant growth [7], Cow dung is a valuable resource for farmers who wish to promote healthy plant growth because of the presence of PGPB. Bacteria like *Bacillus* sp., *Pseudomonas* sp., *Azotobacter* sp., and *Rhizobium* sp. are among the PGPB isolated from cow dung [8]. These bacteria help in nitrogen fixation, solubilization of insoluble phosphorus, potassium solubilization, zinc solubilization,

and siderophore production and make it available to the plants. These PGPB also produce plant growth hormones like auxins, cytokinins, and gibberellins [9]. The PGPB found in cow dung can aid in increasing plant growth and production when it is applied as fertilizer. By boosting nutrient cycling and organic matter content, PGPB can contribute to better soil health in addition to its direct advantages for plants [10]. Long-term increases in soil fertility and productivity may arise from this [8]. Cow dung is a natural and sustainable resource, which has one of its benefits when used as a source of PGPB. In contrast to synthetic fertilizers, which can harm the environment and degrade soil, cow dung can support sustainable agriculture and enhance soil health.

Cow dung is a microbiological pool containing several psychrophilic, mesophilic, and thermophilic bacteria and are responsible for the degradation of complex matter into the simpler ones at different temperature ranges [11]. From cow manure, several *Bacillus* spp. that can produce auxin have been identified [12]. It has been observed that the dung excreted from Indian breeds possesses excellent antimicrobial activity as compared to that of Jersey, Holstein, or buffalo against *K. pneumonia* and *E. coli* [13]. It has been discovered that cow dung and urine exhibit antifungal properties against *Rhizopus stolonifer*, *Sclerotium rolfsii*, and *Fusarium oxysporum* [14, 15].

Organic farming and bioformulations are gaining popularity among farmers and people across the globe for various health benefits and eco-friendly approaches, also to fight against the contrary effects of chemical fertilizers and pesticides without the full knowledge of time and application of the same. Hence, there is urgent need for the scientific validation of cow dung microflora from indigenous and exotic breeds for PGPB. This cow dung is also used as the main input for the preparation of bioformulations [16]. The aim of current study was to isolate and characterize cow dung microflora from indigenous and exotic breeds of cow for various PGP traits, enzyme, and antifungal activities in order to prepare soil inoculants from these potential sources. The bacteria isolated from cow dung can be potential means to protect, enrich, and flourish the soil microbial community as well as plant and soil health [17].

Materials and methods

Sample collection and physicochemical analysis

Samples of cow dung were collected from the Dairy farm of Department of Organic Agriculture and Natural Farming, College of Agriculture, CSK Himachal Pradesh Agricultural University, Palampur, Himachal Pradesh, India, for *Himachali Pahari* lactating (PL), *Himachali Pahari* non-lactating (PD), and *Pahari* bull (PB), whereas Jersey lactating (JL), Jersey non-lactating (JD), Sahiwal lactating (SL), and Sahiwal non-lactating (SD) cow dung samples were collected from the Dairy farm of Department of Livestock Management, College of Veterinary and Animal Husbandry, CSK Himachal Pradesh Agricultural University, Palampur, Himachal Pradesh, India, and brought to the microbiology laboratory in an aseptic manner for further analysis. Physicochemical properties of cow dung, i.e., N, P, K, pH, and EC, were analyzed using standard methods [18–22].

Isolation and enumeration of microorganisms from cow dung

One gram of dung sample was mixed with 9 ml of autoclaved distilled water and mixed well by using vortex mixture. Dilutions of each sample were prepared by using serial dilution method to obtain up to 10^8 dilutions. On the surface of the nutrient agar, 0.1 ml of the diluted sample from each dilution was evenly distributed. The plates were incubated at 28 °C for 24–72 h. The colonies were enumerated, and morphologically distinct colonies were pure cultured and preserved at – 20 °C in glycerol.

Qualitative analysis of PGP traits

Nitrogen fixing ability of bacteria was checked by growing the culture in nitrogen free medium (K_2HPO_4 anhydrous 0.1%, $MgSO_4.7H_2O$ 0.02%, NaCl 0.02%, $FeSO_4$, 0.01%, Na_2MoO_4, 0.0005%, $CaCO_3$ 0.2%, Sucrose 2.0%, Agar 1.5%) [23]. P-solubilization was determined in Pikovskaya's (PVK) medium (glucose 1.0%, Ca_3 $(PO_4)_2$ 0.5%, $(NH_4)_2$ SO_4 0.05%, KCl 0.02%, $MgSO_4.7H_2O$ 0.01%, $MnSO_4$ 0.001%, $FeSO_4$ 0.001%, yeast extract 0.05%, bromocresol purple 0.01%, Agar 1.5%) supplemented with 2% tri-calcium phosphate (TCP) by calorimetric method given by Jackson [21]. Siderophore test was assayed using Chrome-Azural-S (CAS) agar (CAS 0.006%, HDTMA 0.007%, HCl 0.002%, $FeCl_3$ 0.002%, Agar 1.5%) for the production of siderophore [24]. Indole acetic acid (IAA) production was estimated by growing the culture in Luria Bertani broth (yeast extract 0.5%, NaCl 0.5%) supplemented with tryptophan (1.0%) according to the method of Gordon and Weber [25]. Hydrogen cyanide (HCN) production was checked by using the method of Bakker and Schippers [26].

Quantitative analysis of PGP traits

PGP traits, viz., siderophore detection, IAA production, and P-solubilization, were analyzed quantitatively. P-solubilization was analyzed by growing the culture in Pikovskaya's broth containing 0.5% tricalcium phosphate along with appropriate control (uninoculated medium with TCP) at 28 ± 2 °C for 72 h under shaking conditions. The contents were centrifuged at 15,000 rpm for 20 min

at 4 °C. The culture supernatant was used for the determination of soluble phosphate as described by Bray and Kartz [27].

Siderophore detection was checked by first growing the cultures in minimal media (dextrose 0.1%, K_2HPO_4 0.7%, KH_2PO_4 0.2%, sodium citrate 0.05%, $MgSO_4$ 0.01%, $(NH_4)_2SO_4$ 0.1%) along with uninoculated minimal media at 28 ± 2 °C for 48 h. 0.5 ml supernatant was mixed with CAS assay solution (CAS 0.165 gL^{-1}, $FeCl_3$ 0.082 gL^{-1}, and HDTMA 0.397 gL^{-1} in 100 mM piperazine buffer) along with 10 µl of shuttle solution (4 mM 5-sulfosalicylic acid). Mixture was kept at room temperature for 10 min, and absorbance was recorded at 630 nm from change in color from blue to yellow [24].

Quantitative measurement of IAA was done by using calorimetric method of Gorden and Paleg [28] with slight modification. Two to three drops of orthophosphoric acid were added to 2 ml of culture supernatant which was grown in Luria Bertani broth with 5 mM tryptophan and 1% glycerol followed by addition of 4 ml Salper reagent (2 ml of 0.5 M $FeCl_3$ in 98 ml 35% perchloric acid). This mixture was then incubated in dark at room temperature for 25 min. The absorbance was noted at 535 nm for the development of pink color.

Enzyme activity of bacterial isolates

Isolates of cellulolytic bacteria were identified using Teather and Wood's [29] methodology. Carboxymethylcellulose (CMC) agar media (CMC 0.2%, Na_2NO_3 0.1%, K_2HPO_4 0.1%, KCl 0.1%, $MgSO_4$ 0.05%, $FeSO_4$ 0.001%, yeast extract 0.5%, Agar 1.5%) was used to cultivate bacterial isolates for 72 h at 28 ± 2 °C. CMC plates were soaked with iodine solution after growth. A decolorized halo zone around the colony suggested CMC degradation activity.

On skim milk agar plates, the proteolytic activity of each bacterial isolate was examined. Before pouring the plates, 1% skim milk that had been separately sterilized was added to the nutrient agar. Skim milk agar plates were spot-inoculated with bacterial isolates, and the plates were then incubated for 72 h at 28 ± 2 °C. The clear zone formed around the bacterial colony was an indicator of proteolysis.

Amylolytic bacterial isolates were identified by adapting the method of Shaw et al. [30]. Starch agar plates (starch 2%, peptone 0.5%, beef extract 0.3%, Agar 1.5%) were spot inoculated with bacterial isolates and incubated for 72 h at 28 ± 2 °C. After 72 h, plates were flooded with iodine solution. A translucent halo zone around bacterial colony indicated amylolytic activity.

For the estimation of urease activity, 1% bacterial culture was introduced into urea broth (peptone 0.1%, NaCl 0.5%, KH_2PO_4 0.2%, phenol red (0.2% solution) 6 ml, glucose 0.1%, urea (20% aqueous solution) 100 ml) and allowed to incubate at 28 ± 2 °C for 48 h. Observe the broth for a color change and bright pink color of broth indicates urease production.

Antifungal activity of cow dung isolates

To check the antifungal activity of cow dung isolates, agar streak method was incorporated. Bacterial cultures were streaked against *Rhizoctonia solani*, *Sclerotonia sclerotiorum*, *Pythium aphanidermatum*, *Phytopthora* sp., *Alternaria alternata*, and *Fusarium oxysporum* by placing a 5-mm disk in the middle of potato dextrose agar (PDA) (potato 2.0%, dextrose 0.2%, Agar 2.0%) plate along with uninoculated fungal bit as control plate of individual fungal pathogen and incubated at 28 ± 2 °C for 7–10 days.

Identification of bacterial isolates selected on the basis of PGP traits and antifungal activity

The isolates were identified on the basis of morphological, biochemical, and molecular characteristics by using the criteria of Bergey's Manual of Systematic Bacteriology [31].

Morphological characterization

Morphological characteristics of isolates including colony morphology, color, Gram's staining, and cell shape were investigated.

Metabolic fingerprinting

Metabolic fingerprinting was done by using Bergey's manual of systematic bacteriology and commercial kits, i.e., KB009 HiCarbohydrate™ kit.

Molecular taxonomic characterization

(i) Genomic DNA extraction by conventional method

Bacterial isolates were grown for overnight at 35 ± 2 °C in nutrient broth at 200 rpm. 1.5 ml of overnight grown culture was transferred to a micro-centrifuge tube and centrifuged at 12,000 rpm for 1 min, and the supernatant was discarded. Bacterial pellet was suspended in 500 µl of extraction buffer and 50 µl of 10% SDS. Cell pellet was resuspended by vortexing or pipetting and incubate at 65 °C in water bath for 30 min until the sample lysate becomes clear. During incubation, tube was inverted after every 3 min. After incubation, 2 µl of RNase A (50 mg ml^{-1}) was added to sample lysate, mixed by vortexing and then incubated at room temperature for 5 min. Equal volume of phenol to chloroform (1:1) was added to the lysate and mixed well. The above mixture was centrifuged at 10,000 rpm for 5 min at room temperature. Two layers were formed, and the upper aqueous

layer was collected in a new Eppendorf tube with the help of pipette. The phenol to chloroform extraction step was repeated. 1/10 volume of 5 M NaCl and 2.5 volume of absolute ethanol was added to aqueous phase collected in the Eppendorf tube. Incubation of the above mixture was done at − 20 °C for overnight. The above mixture was centrifuged at 12,000 rpm for 20 min at room temperature, and the supernatant was discarded. The DNA pellet was washed with 1 ml of 70% ethanol. DNA pellet was air dried for about 15 min until all the residual ethanol got evaporated. Finally, the DNA pellet was suspended in appropriate amount of TE buffer and quantify [32].

(ii) Characterization of isolates by 16S rRNA gene analysis

PCR was used to specifically amplify 16S rRNA from genomic DNA, using genus specific primers of 27F 5′ (AGA GTT TGA TCC TGG CTC AG) 3′ and 1492R 5′ (TAC GGT TAC CTT GTT ACG ACT T) 3′ for 14 bacterial isolates. The following temperature profile was used for DNA amplification: an initial denaturation step of 94 °C for 3 min followed by 35 cycles of 94 °C for 1 min, annealing temperature of 60 °C for 30 s and 72 °C for 1 min 30 s, and final extension step of 72 °C for 10 min. To eliminate any risk of contamination from extraneous DNA, the reaction mixture, without the template, was simultaneously run along with PCR reaction as a control. The amplified PCR product of bacterial isolate was resolved by electrophoresis using 1.0% agarose gel in 1X Tris–acetate EDTA buffer containing ethidium bromide (0.5 µl ml^{-1}). DNA ladder of 100 bp was used as marker. The gel was run at 120 V for 2 h using Bangalore Genei power system. The gel documentation system (Genei) was used to view the gels and take pictures. To counter possible stochastic effects of PCR [33], five amplifications were carried out on each sample and pooled prior to purification and cloning. Amplified PCR products were eluted from the gel using gel extraction kit (Hi Yield Gel/PCR DNA Extraction Kit from Real Genomics); eluted fragment was then sequenced using PCR primers. Using BLAST, the sequence was aligned with matched 16S rDNA sequences from the database [34]. Multiple alignments were generated by the MULTALIN program [35]. The Molecular Evolutionary Genetics Analysis (MEGA X) program was used to build the phylogenetic tree [36]. Tree was viewed with the help of TreeView [37].

Pot experiment on pea crop

The experiment was set up at the Natural Farm of Department of Organic Agriculture and Natural Farming, College of Agriculture, CSK Himachal Pradesh Agricultural University, Palampur, Himachal Pradesh, India. The experiment was conducted with 15 treatments including one control. The soil was passed through a 2-mm sieve before being used in a pot culture experiment. To create the potting mixture, sand, soil, and farm yard, manure was put together in a 1:1:1 ratio. After that, the mixture was poured into the pots and moistened to one third of its capacity. In fourteen treatments, four pea seeds were sown in each pot and inoculated with individual selected cow dung isolate (20 ml of 1 O.D containing 10^7 cfu ml^{-1}) along with one uninoculated treatment to see the effect of microbes on soil and growth parameters of pea plants. The plants were treated with 20 ml of bacterial culture during sowing, and reinoculation was done after 30 days interval up to 60 days to see the effect of microbes on plant height, germination percent, days to 1st flowering, pods per plant, microbial count of rhizospheric soil, and N, P, and K content of soil after completing the experiment. Initial data of microbial count, N, P, and K of composite soil used for pot experiment was also taken and mentioned under the "Results and discussion" section.

The treatment details for the experiment are given below:

Number of treatments	15
Crop	Pea
Variety	PB 89
Date of sowing	20–01–2024

Treatments	Inoculation with cow dung isolates
T1	PL2 (*Stenotrophomonas maltophilia*)
T2	PL3 (*Microbacterium thalassium*)
T3	PD3 (*Bacillus subtilis*)
T4	PD5 (*Bacillus subtilis*)
T5	SL1 (*Bacillus subtilis*)
T6	SL2 (*Bacillus* sp.)
T7	SL5 (*Arthrobacter gandavensis*)
T8	SD3 (*Escherichia coli*)
T9	JL1 (*Bacillus subtilis*)
T10	JL2 (*Bacillus subtilis*)
T11	JL3 (*Escherichia coli*)
T12	JL4 (*Bacillus subtilis*)
T13	JD1 (*Bacillus subtilis*)
T14	JD2 (*Bacillus licheniformis*)
T15	Control

Statistical analysis

All the experiments were conducted in triplicates along with equal number of appropriate controls. For screening and comparison of various treatments, the data was subjected to analysis of various technique (ANOVA) using completely randomized design (CRD). The means were

Table 1 Physicochemical parameters of cow dung from different breeds of cow

Sr. no.	Cow dung samples	pH	N%	P%	K%	EC (mSm $^{-1}$)
1	***Himachali Pahari* lactating**	5.33	0.357	0.119	0.865	5.41
2	***Himachali Pahari* non-lactating**	5.26	0.369	0.124	0.892	5.22
3	**Sahiwal lactating**	4.83	0.346	0.113	0.858	6.52
4	**Sahiwal non-lactating**	5.13	0.349	0.117	0.860	5.43
5	**Jersey lactating**	5.30	0.336	0.106	0.846	4.43
6	**Jersey non-lactating**	4.93	0.340	0.109	0.848	4.23
7	***Pahari* bull**	5.60	0.354	0.117	0.864	6.82
	SE (m) ±	0.060	0.001	0.001	0.001	0.035
	LSD (p <0.05)	0.185	0.002	0.003	0.003	0.106

SE (m) ± standard error mean

LSD least significant difference

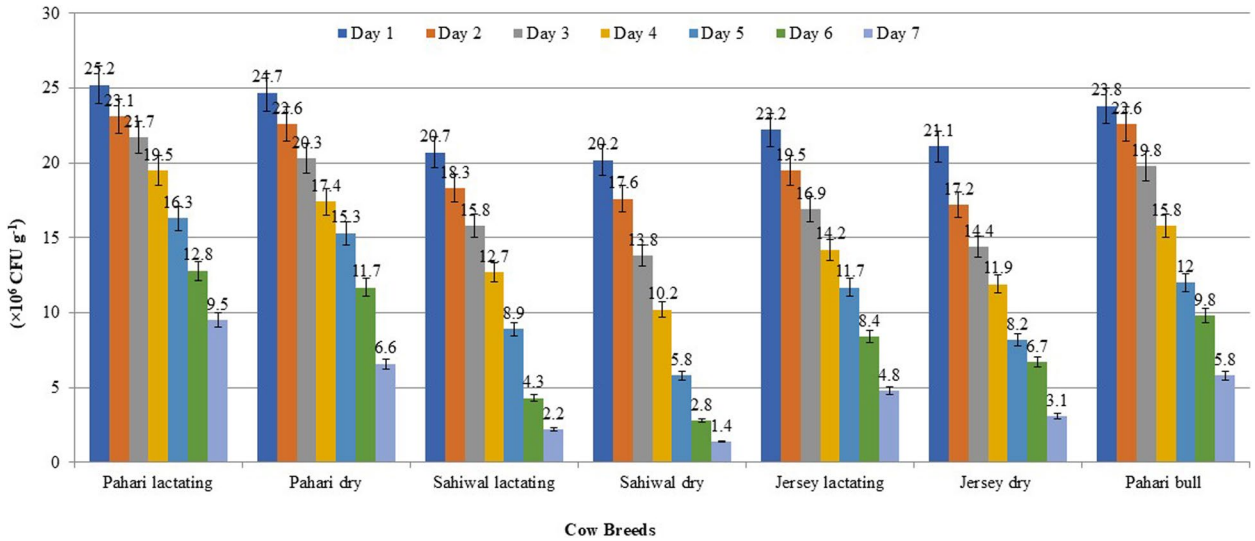


Fig. 1 Enumeration of microbial population of cow dung of different breeds on nutrient agar media (× 10^6 CFU/g)

compared with the least significant difference (LSD), and the levels of significance were represented with the *p*-value significance level. The data was analyzed by using SPSS version 29.

Results and discussion

Physicochemical parameters of cow dung from different breeds of cow

Physicochemical properties namely, pH, EC, N, P, and K of cow dung from different breeds of cow were analyzed. A perusal of data presented in Table 1 shows that highest value of pH (5.60) was recorded in the dung of *Pahari* bull, while significantly higher values (p <0.05) of N, P, and K (0.369%, 0.124%, 0.892%) were observed in dung of *Himachali Pahari* non-lactating, respectively, and it was found to be statistically at par (p <0.05) with *Himachali Pahari* lactating (0.357%, 0.119%, 0.865%). Lowest values of N, P, and K were recorded in the dung of Jersey lactating. Significantly (p <0.05), the highest value of EC was recorded in cow dung of *Pahari* bull followed by dung of Sahiwal lactating, whereas the lowest value was observed in Jersey non-lactating (Table 1).

The results for physicochemical properties have been found to be in contrast as compared to the findings of Bhatt and Maheshwari [38], where the values of pH, N, P, and K were higher except EC.

Isolation and enumeration of microorganisms from dung of different breeds of cow

A total of one thousand two hundred and sixty-three (1263) bacteria were isolated from dung of six different cows and one bull. Maximum microbial count of cow dung from different cow breeds and bull was found on first day. Microbial count was found maximum in *Himachali Pahari* lactating (25.2 × 10^6 cfu g^{-1}) followed by *Himachali Pahari* non-lactating (24.7 × 10^6 cfu g^{-1}),

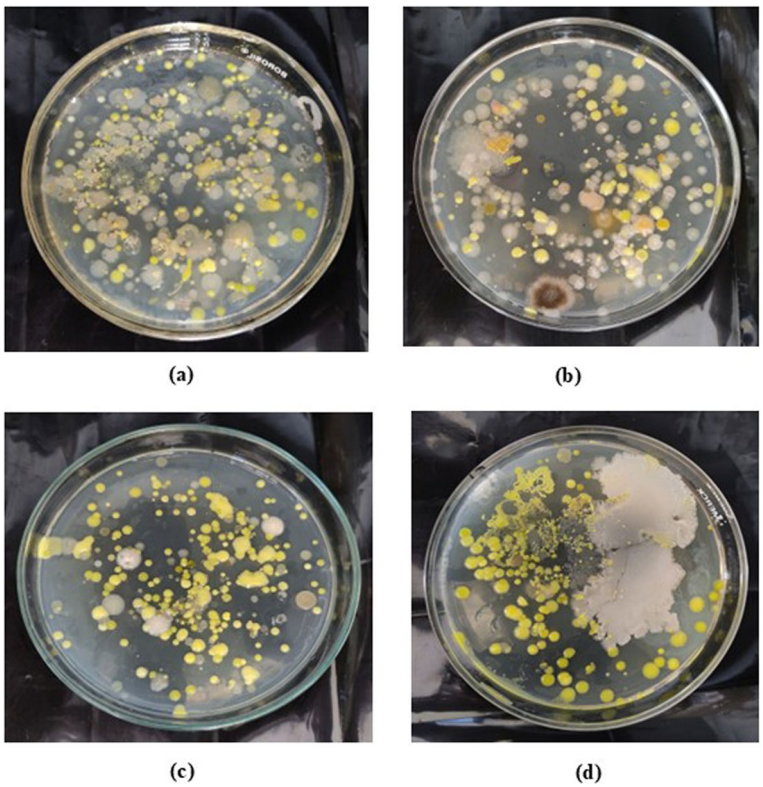


Fig. 2 Microbial colonies present in cow dung of different breeds: (**a**)*Himachali Pahari* lactating ($\times 10^6$ CFU g^{-1}), (**b**) Jersey lactating ($\times 10^6$ CFU g^{-1}), (**c**) Sahiwal lactating ($\times 10^6$ CFU g^{-1}), (**d**)*Pahari* bull ($\times 10^6$ CFU g^{-1})

whereas minimum microbial count was found in Sahiwal non-lactating (20.2×10^6 cfu g^{-1}) as shown in Fig. 1. Figure 2 showed the microbial colonies isolated from cow dung of different breeds.

The results have been in contrast with the findings of Ajunwa et al. [39] where the microbial count has been found to be increasing from day 1 (3.5×10^6 cfu g^{-1}) to day 10 (5.1×10^8 cfu g^{-1}) and continued to do so until day 30 (4.1×10^4 cfu g^{-1}). Sharma and Singh [5] have isolated bacteria from desi cow and it has been found that there were significant larger number of bacteria present in cow dung even at 10^7 dilutions, i.e., 24.0×10^5 cfu g^{-1} in sample 1, 20.0×10^5 cfu g^{-1} in sample 2, and 15.0×10^5 cfu g^{-1} in sample 3. Godambe and Fulekar [40] have also enumerated total viable cells in cow dung, i.e., 2.29×10^8 cells ml^{-1}. The microbial count in present study is lower than that of the findings of other authors which may be because of the climatic conditions of the study area, diet difference in cattle feed, and gut microbiota of the bovine animal.

Qualitative analysis of PGP traits

Thirty-two morphologically different microbes were screened for various PGP traits, i.e., nitrogen fixing ability, P-solubilization, IAA production, siderophore production, and HCN production. Different PGP traits shown by bacterial isolates of cow dung of different breeds is presented in Table 2, and a plate showing different PGP traits is presented in Fig. 3. Out of these thirty-two bacterial isolates screened for PGP activities, twenty-two isolates were found positive for nitrogen fixation, twenty-seven were found positive for P-solubilization, twenty showed siderophore production, twenty-seven were found positive for IAA production, and none were found positive for HCN production. Percent of cow dung isolates of different breeds showed PGP traits showed in Fig. 4. Results revealed that 11 bacterial isolates, i.e., PL2, PL3, PD3, PD5, SL1, SL2, SL5, SD3, JL1, JD1, and JD2, exhibited concomitant production of all plant growth-promoting activities except for HCN production (Table 2).

Similar findings in relation to PGP traits of bacteria from the same source has also been described [41, 42] except for HCN production. HCN is not produced by all plant growth-promoting rhizobacteria. Many bacterial genera have the ability to produce HCN because HCN production is independent of genus [43]. Lotfi et al. [44] found that no strain was found to have the capability of

Table 2 Screening of bacterial isolates for multifarious plant growth-promoting activities by qualitative assay method

Bacterial isolates	Nitrogen fixing ability	Phosphate solubilization	Siderophore production	IAA production	HCN production
PL1	+ +	+ + +	–	+	-
PL2	+ + +	+ + +	+	+ +	-
PL3	+ +	+ + +	+	+ +	-
PL4	–	+ + +	+	+	-
PD1	+ +	–	–	+	-
PD2	+ + +	–	–	+	-
PD3	+ +	+ + +	+ +	+	-
PD4	+ +	+ + +	+	–	-
PD5	+ + +	+ + +	+	+ +	-
SL1	+ +	+ + +	+ +	+	-
SL2	+ + +	+ + +	+ +	+	-
SL3	+ +	+ + +	–	+ + +	-
SL4	-	+ + +	+	+ + +	-
SL5	+ +	+ + +	+	+	-
SD1	+ +	+ + +	–	+ + +	-
SD2	-	+ + +	–	–	-
SD3	+ +	+ + +	+	+ + +	-
SD4	-	+ + +	–	–	-
SD5	+ +	+ + +	–	+ + +	-
SD6	-	+ + +	–	+	-
JL1	+ +	+ + +	+	+ +	-
JL2	+ +	+ + +	+ +	–	-
JL3	+ + +	+ + +	–	+ + +	-
JL4	-	–	+	–	-
JD1	+ +	+ + +	+ +	+ +	-
JD2	+ +	+ + +	+ +	+ +	-
JD3	-	+ + +	+	+ + +	-
JD4	-	+ + +	+	+ +	-
PB1	+ +	–	+ +	+	-
PB2	–	–	+	+ +	-
PB3	+ +	+ + +	–	+ + +	-
PB4	–	+ + +	–	+ + +	-
Total	22	27	20	27	0

+ Fair

+ + Good

+ + + Very good

PL Himachali Pahari lactating, *PD Himachali Pahari* non-lactating, *SL* Sahiwal lactating, *SD* Sahiwal non-lactating, *JL* Jersey lactating, *JD* Jersey non-lactating, *PB Pahari* bull

producing HCN but producing siderophore, IAA, and gibberellic acid. Cow dung isolates showed P-solubilization and IAA production [41]. The bacteria isolated from cow dung have also shown potential to be used as PGPB [42]. Thus, the results imply that cow dung bacteria are potential plant growth promoters and help in positive plant growth promotion directly by fixing nitrogen, solubilizing phosphorous and making it available for plants and the IAA production for plant growth as well as indirectly through siderophore production and HCN production.

Quantitative analysis of plant growth-promoting traits

Twenty isolates for P-solubilization, twenty-seven isolates for IAA production, and nineteen isolates for siderophore production were analyzed for quantitative estimation on the basis of qualitative assay. Perusal of data showed in Table 3 revealed that the individual

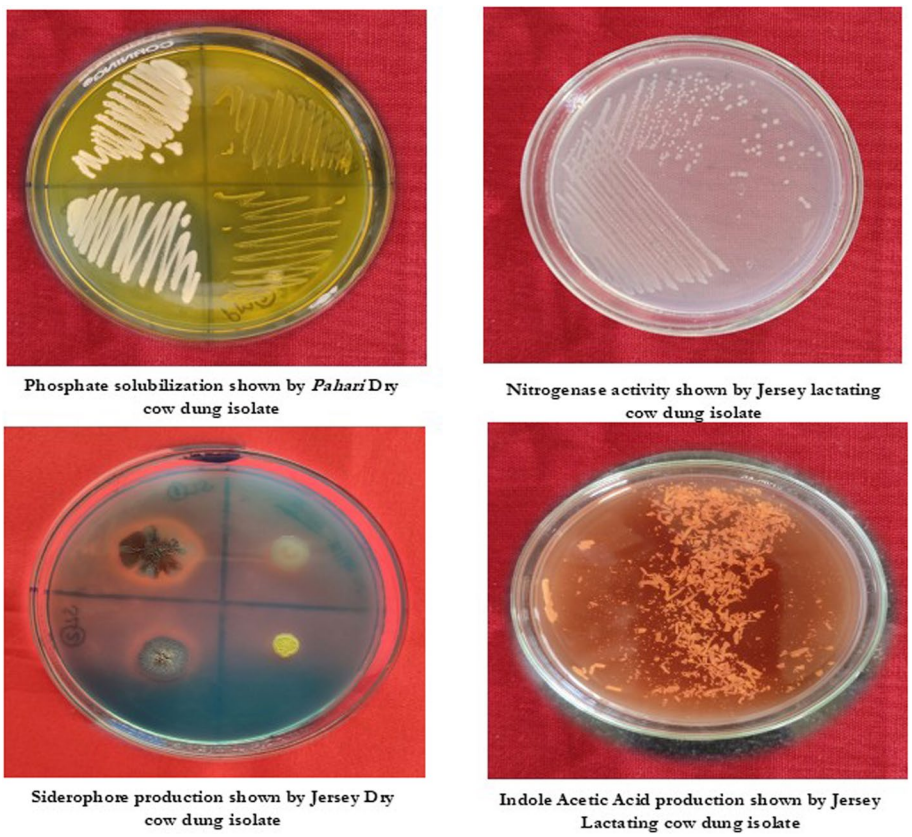


Fig. 3 Plates showing PGP traits by bacterial isolates of cow dung

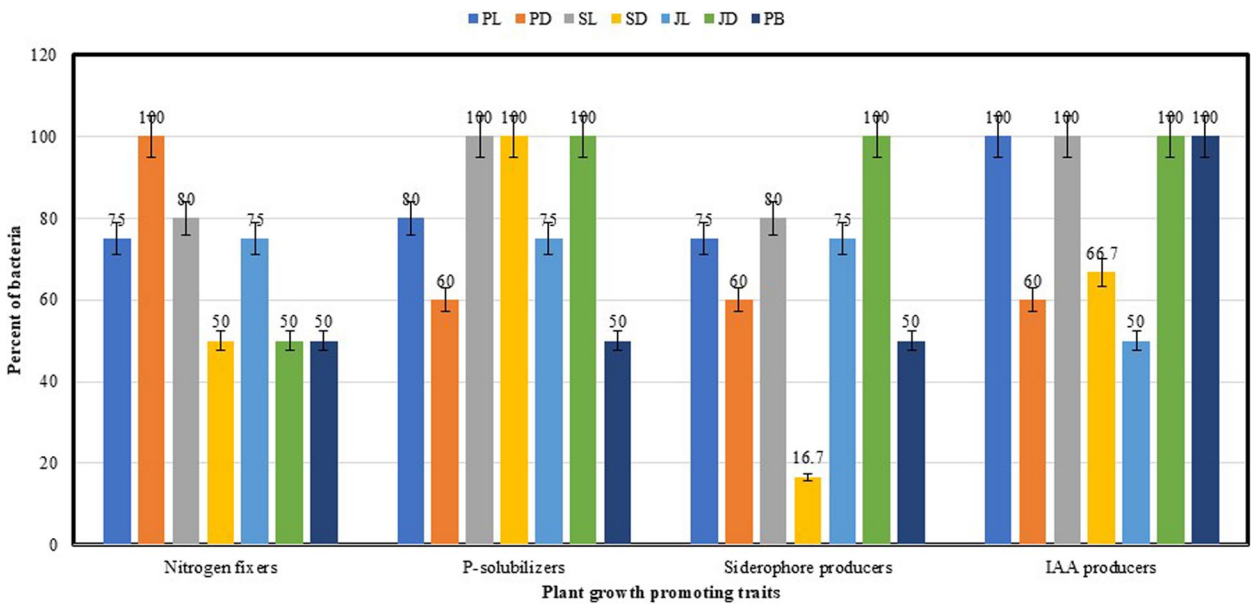


Fig. 4 Percent of cow dung isolates showing plant growth-promoting traits

Table 3 Quantitative estimation of phosphate solubilization, indole acetic acid production, and siderophore production

Bacterial isolates	Phosphate solubilization (µg/ml)	IAA production (µg/ml)	Siderophore production (% siderophore unit)
PL1	13.49	8.01	-
PL2	13.00	80.03	86.61
PL3	65.39	20.26	94.49
PL4	35.36	5.43	71.65
PD1	-	2.73	-
PD2	-	6.55	-
PD3	65.46	76.33	91.34
PD4	61.88	-	97.37
PD5	92.72	13.40	94.49
SL1	55.39	43.18	64.57
SL2	61.52	18.46	63.78
SL3	104.91	47.56	-
SL4	35.36	8.35	0
SL5	78.49	9.58	94.49
SD1	78.39	-	-
SD2	13.92	-	-
SD3	68.07	53.18	63.78
SD4	12.51	-	-
SD5	105.82	44.75	-
SD6	15.04	15.20	-
JL1	45.68	58.69	46.46
JL2	56.59	-	90.55
JL3	69.06	46.33	-
JL4	-	-	58.27
JD1	33.35	39.02	72.44
JD2	53.00	18.80	-
JD3	127.79	55.65	96.85
JD4	70.98	5.09	98.42
PB1	-	75.20	58.27
PB2	-	8.12	77.95
PB3	14.20	4.42	-
PB4	14.55	5.09	-

PL Himachali Pahari lactating, *PD Himachali Pahari* non-lactating, *SL* Sahiwal lactating, *SD* Sahiwal non-lactating, *JL* Jersey lactating, *JD* Jersey non-lactating, *PB Pahari* bull

bacterial isolate effectively solubilizes the insoluble tri calcium phosphate and produced a considerable amount of IAA and siderophores in liquid medium. Maximum P-solubilization (127.79 µg ml^{-1}) was recorded for isolate JD3 followed by 105.82 µg ml^{-1} soluble phosphate by bacterial isolate SD5 and 104.91 µg ml^{-1} by isolate SL3. The isolate SD4 solubilized minimum TCP with the release of 12.51 µg ml^{-1} phosphorus.

The results for P-solubilization are in similarity with that of Radha and Rao, Pandey et al., and Bhatt and Maheshwari [38, 45, 46]. Bhatt and Maheshwari [38] examined cow dung microbes for the production of various plant growth-promoting traits. The bacterial isolates were analyzed for the production of phosphate. Out of selected two bacterial isolates, CDK25 had the highest nutritional solubility (281.59 mg ml^{-1}), followed by CDK15 (264.04 mg ml^{-1}). P-solubilization by *Bacillus* sp. has also been described by Dubey et al. [47] who demonstrated P-solubilization by *Bacillus subtilis* (BSK17), in addition to increasing *Cicer arietinum* yield. Thus, it can be inferred that cow dung bacteria possess the ability to mobilize a higher amount of phosphate for it to be made available for plants.

The bacterial isolate JD4 produced maximum siderophore unit (98.42%) at 72 h of incubation followed by PD4 (97.37%), JD3 (96.85%), PD5 (94.48%), and SL5 (94.48%). Minimum percent siderophore unit (46.46%) was found in case of isolate JL1. In order to absorb iron from their surroundings and then provide it to plants to reduce iron stress, bacteria and fungus make siderophores, which are iron chelating agents. These kinds of bacteria can be a potential inoculant to be used in fields and agriculture soils. Karnwal [48] have isolated PGP bacteria from cow dung which were able to produce siderophore to alleviate iron stress.

Cow dung isolate PL2 produced higher concentration of IAA (80.03 µg ml^{-1}) followed by isolate PB1 (75.20 µg ml^{-1}) after 72 h of incubation as compared to other cow dung bacterial isolates. Minimum IAA (2.73 µg ml^{-1}) was recorded for bacterial isolate PD1. The results for IAA production coincide with the findings of Bhatt and Maheshwari [38] and Dubey et al. [47]. Bhatt and Maheshwari [38] tested two cow dung bacteria for the production of IAA, and it has been observed that both isolates produced IAA with the range of 13.8 µg ml^{-1} by CDK25 and 11.6 µg ml^{-1} by CDK15. As a result, there was an increase in the vegetative growth parameters. Similarly, Radha and Rao [45] also reported IAA production by cow dung bacteria. Pandey et al. [46] also isolated IAA producing *B. subtilis* and *B. pumilus* at the rate of 9.5 µg ml^{-1} and 7.9 µg ml^{-1}, respectively. IAA production was also reported by Gontia-Mishra et al. [49] with a range of 4.7–77.41 µg ml^{-1}.

Enzyme production

The potential 32 bacterial isolates were further evaluated for their enzymes production. In the present study, cellulase activity was exhibited by only 11 isolates, viz., PL3, PD3, SL1, SL2, SD2, JL1, JL2, JL4, JD1, JD2, and JD4 on CMC media amended with 0.2 percent powdered carboxymethylcellulose, with the production of halo zones of cellulose degradation ranging from 0.2 to 2.52 cm (Table 4; Fig. 5). Among all 11 isolates, maximum halo

Table 4 Enzyme activity of bacteria isolated from cow dung of different breeds

Bacterial isolates	Urease	Amylase (zone size in cm)[a]	Protease (zone size in cm)[a]	Cellulase (zone size in cm)[a]
PL1	-	-	-	-
PL2	-	-	+(0.6)	-
PL3	-	-	-	+(0.23)
PL4	-	+++(3.13)	+++(2.3)	-
PD1	-	-	-	-
PD2	-	++(1.2)	-	-
PD3	-	-	+++(2.7)	+++(2.18)
PD4	-	-	-	-
PD5	-	-	-	-
SL1	-	-	-	++(1.36)
SL2	+	+++(3.0)	-	++(1.3)
SL3	-	-	-	-
SL4	-	-	-	-
SL5	-	-	-	-
SD1	-	-	-	-
SD2	-	-	-	+(0.2)
SD3	-	+(0.8)	-	-
SD4	-	+(0.6)	-	-
SD5	-	-	-	-
SD6	-	-	-	-
JL1	-	+++(2.47)	++(1.43)	+++(2.13)
JL2	-	+++(2.3)	-	++(1.5)
JL3	-	-	-	-
JL4	-	+++(3.1)	+++(2.06)	++(1.53)
JD1	-	+++(3.4)	-	+++(2.52)
JD2	-	++(1.6)	-	++(1.63)
JD3	-	-	-	-
JD4	-	-	-	++(1.1)
PB1	-	-	-	-
PB2	-	-	-	-
PB3	+	-	+(0.5)	-
PB4	-	-	-	-

[a] +: 0–1 cm
++: 1–2 cm
+++: > 2 cm

PL Himachali Pahari lactating, *PD Himachali Pahari* non-lactating, *SL* Sahiwal lactating, *SD* Sahiwal non-lactating, *JL* Jersey lactating, *JD* Jersey non-lactating, *PB Pahari* bull

zone for cellulase enzyme was observed in case of the isolate JD1.

Among 32 bacterial isolates, 2 isolates (SL2 and PB3) were able to degrade urea with the production of urease enzyme. Out of 32 isolates, 10 isolates were able to produce amylase enzyme for the degradation of starch, viz., PL4, PD2, SL2, SD3, SD4, JL1, JL2, JL4, JD1, and JD2 on starch agar amended with 2% soluble starch with the

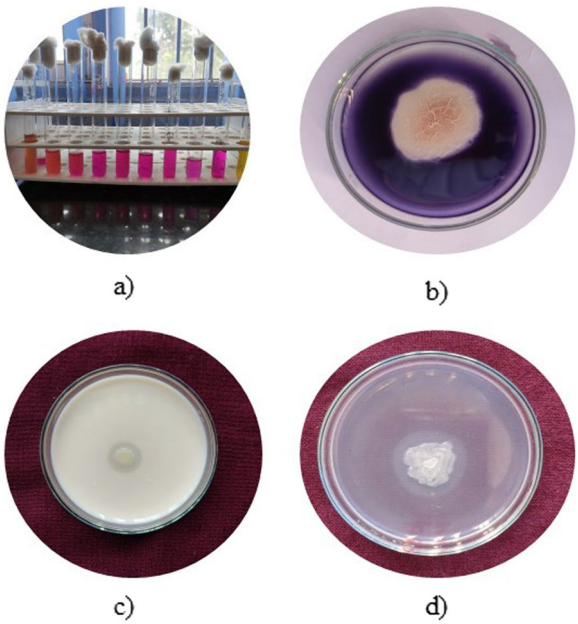


Fig. 5 Enzymatic activity by bacterial isolates: (**a**) urease activity by SL2 isolate, (**b**) amylase activity by PL4 isolate, (**c**) protease activity by JL1 isolate, (**d**) cellulase activity by JL1 isolate

production of halo zones around the colony ranges from 0.6 to 3.4 cm. Six isolates showed protease activity, viz., PL2, PL4, PD3, JL1, JL4, and PB3 with the utilization of casein protein present in skim milk by producing the halo zones around the colony in the range of 0.5 to 2.06 cm, respectively. Except for isolate PL1, PL3, PD1, PB1, PB2, and PB4, all other 27 isolates were able to produce phosphatase (Table 4; Fig. 5).

Enzyme activity of bacterial isolates showed that bacteria were able to produce cellulase, amylase, urease, phosphatase, and protease. Cow dung has been found to be actively degrading starch by amylase activity [50]. Cellulase producing alkaline bacteria has also been isolated from cow dung [51]. Sharma and Singh [5] have also found that indigenous cow dung bacterial isolates possess enzyme activity, viz., protease, amylase, and lipase activity. All these similar studies support that cow dung bacteria possess enzyme activity and are able to metabolize complex substances with the release of extracellular enzymes.

Antifungal activity of bacterial isolates

Using the dual culture approach, the antifungal activity of individual isolates was compared. The individual isolates showed a varied level of antifungal activity against six fungal phytopathogens namely; *Sclerotinia sclerotiorum*, *Pythium aphanidermatum*, *Phytophthora* sp.,

Table 5 Antifungal activity of cow dung bacterial isolates against *Sclerotinia sclerotiorum*, *Pythium aphanidermatum*, *Phytophthora* sp., *Fusarium oxysporum*, *Rhizoctonia solani*, and *Alternaria alternata* using agar streak method

Bacterial isolates	Percent growth inhibition against different fungal pathogens					
	***Sclerotinia sclerotiorum* (ITCC no. 6094)[b]**	***Pythium aphanidermatum* (ITCC no. 8017)**	***Phytophthora* sp. (ITCC no. 7700)**	***Fusarium oxysporum* (ITCC no. 8634)**	***Rhizoctonia solani* (ITCC no. 7855)**	***Alternaria alternata* (ITCC no. 6055)**
PL1	-	-	-	-	-	-
PL2	CI[a]	50.00	CI	-	-	CI
PL3	-	-	-	-	-	-
PL4	-	-	-	-	-	-
PD1	-	47.36	-	-	-	-
PD2	-	-	-	-	-	-
PD3	-	-	-	-	-	-
PD4	-	-	-	-	-	-
PD5	-	CI	-	-	-	-
SL1	78.26	73.68	CI	CI	CI	-
SL2	CI	47.36	-	-	-	72.09
SL3	-	60.52	CI	-	-	-
SL4	-	-	-	-	-	-
SL5	-	76.31	-	-	-	-
SD1	CI	-	CI	-	CI	CI
SD2	-	-	-	-	-	-
SD3	CI	-	CI	-	CI	CI
SD4	-	-	-	-	-	CI
SD5	CI	-	CI	-	CI	-
SD6	-	-	-	-	-	-
JL1	69.56	50.00	CI	CI	CI	90.69
JL2	89.13	76.31	CI	55.55	CI	67.44
JL3	-	57.89	50.00	-	-	-
JL4	69.56	78.94	CI	CI	CI	55.81
JD1	84.78	76.31	CI	CI	CI	CI
JD2	CI	52.63	-	-	CI	86.04
JD3	-	-	-	-	-	-
JD4	-	-	-	-	-	-
PB1	-	-	-	-	-	-
PB2	-	-	-	-	-	-
PB3	-	CI	-	-	-	-
PB4	-	-	-	-	-	-

[a] *CI* contact inhibition

[b] *ITCC* Indian Type Culture Collection

PL Himachali Pahari lactating, *PD Himachali Pahari* non-lactating, *SL* Sahiwal lactating, *SD* Sahiwal non-lactating, *JL* Jersey lactating, *JD* Jersey non-lactating, *PB Pahari* bull

Fusarium oxysporum, *Rhizoctonia solani*, and *Alternaria alternate* (Table 5).

By using the dual culture approach, only 17 of the 32 bacterial isolates from various cow breeds exhibited antifungal activity against one or more fungal phytopathogens (Fig. 6). As shown in Table 5, out of 4 bacteria isolated from *Himachali Pahari* lactating, only PL2 exhibited contact inhibition against *S. sclerotiorum*, *Phytophthora* sp., and *A. alternata* and clear zone inhibition (50%) against *P. aphanidermatum*. Two isolates from PD1 and PD5 from *Himachali Pahari* non-lactating showed clear zone inhibition (47.36%) and contact inhibition against *P. aphanidermatum*, respectively. In case of Sahiwal lactating except for SL4 isolate, other three isolates SL1, SL2, and SL3 showed antifungal activity against fungal pathogens. SL1 showed clear

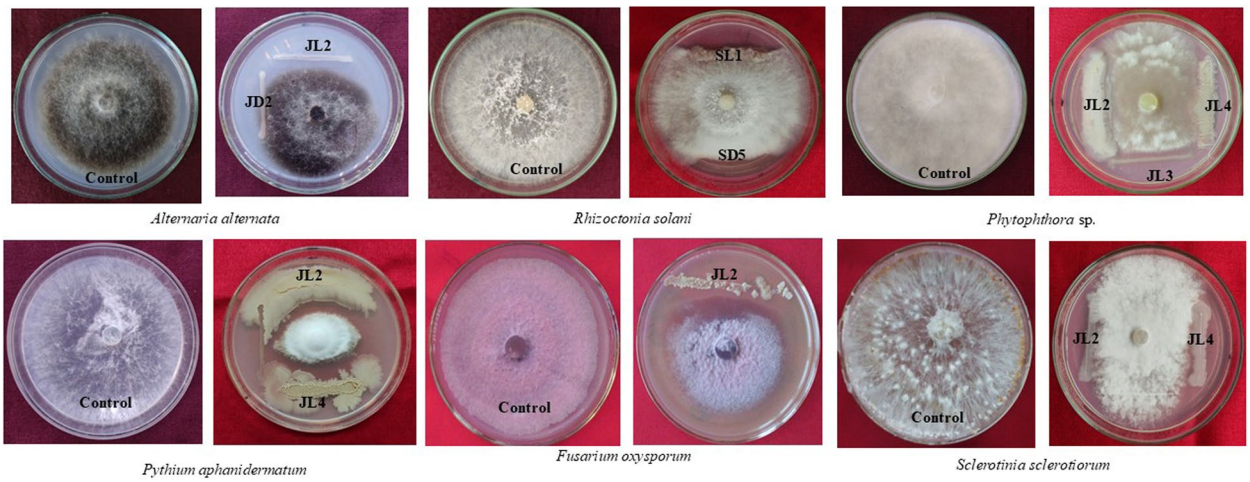


Fig. 6 Antifungal activity by bacterial isolates against different fungal pathogens

zone inhibition against *S. sclerotiorum* (78.26%) and *P. aphanidermatum* (73.68%), whereas it was contact inhibition against *Phytophthora* sp., *F. oxysporum*, and *R. solani*. No inhibition was found against *A. alternata*. SL2 showed contact inhibition against *S. sclerotiorum* and clear zone inhibition against *P. aphanidermatum* (47.36%) and *A. alternata* (72.09%). SL3 showed clear zone inhibition against *P. aphanidermatum* (60.52%) and contact inhibition against *Phytophthora* sp.

In case of Sahiwal non-lactating isolates, SD1, SD3, SD4, and SD5 have showed contact inhibition against fungal pathogens as mentioned in Table 5. SD2 and SD6 were unable to show antifungal activity against any of the fungal pathogens. Among Jersey lactating isolates, JL1, JL2, and JL4 have showed either clear zone inhibition or contact inhibition against all the six fungal pathogens studied, whereas JL3 showed clear zone inhibition against *P. aphanidermatum* (57.89%) and *Phytophthora* sp. (50.00%). JL1 isolate showed maximum clear zone inhibition against *A. alternata* (90.69%), JL2 isolate showed maximum clear zone inhibition against *P. aphanidermatum* (78.94%), and JL4 showed maximum inhibition against *S. sclerotiorum* (89.13%). Two of the four isolate from Jersey non-lactating showed antifungal activity against fungal pathogens. JD1 isolate showed clear zone inhibition against *S. sclerotiorum* (84.78%) and *P. aphanidermatum* (76.31%), whereas contact inhibition against other four fungal pathogens. JD2 showed contact inhibition against *S. sclerotiorum* and *R. solani*, whereas clear zone of inhibition against *P. aphanidermatum* (52.63%) and *A. alternata* (86.04%). Out of four *Pahari* bull isolates, only PB3 showed contact inhibition against *P. aphanidermatum*.

Cow dung isolates possess antifungal activity against six fungal pathogens used in the study. Some isolates showed contact inhibition, where some of the isolates showed clear zone inhibition. The results are similar to the findings of Ram et al. [41]. Meena et al. [52] has also shown the same results with cow dung as a binder being 36.36% effective against *Alternaria*. Cow dung at 2.5, 5.0, and 7.5% have been found effective against *A. alternata* with mycelial growth inhibition of 86.23%, 83.12%, and 67.23%, respectively [53]. Soil amendments with the mixture of cow dung has been found effective against *S. sclerotiorum* [54].

Identification of selected cow dung bacterial isolates on the basis of plant growth-promoting enzyme and antifungal activities

Morphological features of cow dung bacterial isolates

The results indicate colony morphology, Gram's staining, and cell shape of bacterial isolates from cow dung (supplementary material, Table S1). Eleven bacterial isolates were found to be rods, two were coccobacilli, and only one was cocci in shape. Most of the bacterial isolates were circular, some are irregular, and very few are punctiform in shape. Color of the colonies varied from white to yellow. Eleven isolates were found to be gram positive, whereas three were found to be gram-negative bacteria. These results are in partial agreement with the findings of Radha and Rao [45], where all the seven bacterial strains isolated were gram positive rods and none were found gram negative that exhibited white to off white colony color in agar plates.

Table 6 Biochemical characterization of bacteria isolated from cow dung of different breeds

No.	Test	PL2	PL3	PD3	PD5	SL1	SL2	SL5	SD3	JL1	JL2	JL3	JL4	JD1	JD2
1	Catalase	+[a]	+	+	+	+	+	+	-	+	+	-	+	+	+
2	Cellulase	-[b]	+	+	-	+	+	-	-	+	+	-	+	+	+
3	Protease	+	-	+	-	-	-	-	-	+	-	-	+	-	-
4	Starch hydrolysis	-	-	-	-	-	+	-	+	+	+	-	+	+	+
5	Lactose	-	+	-	-	-	-	-	-	-	-	+	-	-	-
6	Xylose	-	+	-	-	-	-	-	+	-	-	+	-	-	-
7	Maltose	-	+	-	-	-	-	-	+	-	-	+	-	-	-
8	Fructose	-	+	-	-	-	-	-	+	-	-	+	-	-	-
9	Dextrose	-	+	-	-	-	-	-	+	-	+	+	-	-	-
10	Galactose	-	+	-	-	-	-	-	+	-	-	+	-	-	-
11	Raffinose	-	-	-	-	-	-	-	-	-	-	+	-	-	-
12	Trehalose	-	+	-	-	-	-	-	+	-	-	+	-	-	-
13	Melibiose	-	-	-	-	-	-	-	-	-	-	+	-	-	-
14	Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	L-arabinose	-	+	-	-	-	-	-	+	-	-	+	-	-	-
16	Mannose	-	+	-	-	-	-	-	+	-	-	+	-	-	-
17	Inulin	-	-	+	-	-	-	-	-	-	-	-	-	-	-
18	Sodium gluconate	-	+	-	-	-	-	-	+	-	-	+	-	-	-
19	Glycerol	-	+	-	-	-	-	-	-	-	-	-	-	-	-
20	Salicin	-	-	-	-	+	-	-	+	-	-	-	-	-	-
21	Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	Sorbitol	-	-	-	-	-	-	-	-	-	+	+	-	-	-
24	Mannitol	-	-	-	-	-	-	-	+	-	-	+	-	-	-
25	Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	α/methyl/D/glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	Rhamnose	-	+	-	-	-	-	-	-	-	-	+	-	-	-
30	Cellobiose	-	-	-	-	+	-	-	-	-	-	-	-	-	-
31	Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	α/methyl/D/mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	ONPG	-	+	+	-	-	+	-	+	+	+	+	+	+	+
35	Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+
36	D-arabinose	-	+	-	-	-	-	-	+	-	-	+	-	-	+
37	Citrate utilization	+	-	+	-	-	-	-	+	+	+	+	-	-	-
38	Malonate utilization	+	-	-	-	-	-	-	-	-	-	-	-	-	-
39	Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40	Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] +: positive reaction

[b] -: negative reaction

Biochemical characterization of cow dung bacterial isolates

The physiological and biochemical characteristics of the bacterial isolates are given in Table 6. The isolates were aerobic, catalase-producing strains except SD3 and JL3. Starch was hydrolyzed through production of enzyme amylase by isolates SL2, SD3, JL1, JL2, JL4, JD1, and JD2. The strains were positive for cellulolytic except for PL2, PD5, SL5, SD3, and JL3 and could utilize a wide array of carbohydrates (assessed using a KB009 Hicarbohydrate™ Kit), including lactose (PL3, Jl3), xylose (PL3, SD3 and JL3), maltose (PL3, SD3 and JL3), fructose (PL3, SD3 and JL3), dextrose (PL3, SD3,

JL2 and JL3), galactose (PL3, SD3 and JL3), trehalose (PL3, SD3 and JL3), L-arabinose (PL3, SD3 and JL3), mannose (PL3, SD3 and JL3), inulin (PD3), sodium gluconate (PL3, SD3 and JL3), glycerol (PL2), salicin (SL1 and SD3), cellobiose (SL1), sorbitol (JL2 and JL3), mannitol (SD3 and JL3), rhamnose (PL2 and JL3), and D-arabinose (PL3, SD3, JL3 and JD2), as sole carbon source. All strains were able to hydrolyze esculin and except for PL2, PD5, SL1, SL2, and SL5; all strains were positive for ONPG test. The results on the utilization of carbon sources were in conformity with the earlier reports on the catabolic ability of *B. subtilis* and *B. cereus* [55]: *B. licheniformis* [56] and *B. safensis* [57].

Molecular characterization and phylogenetic analysis

16S rRNA gene sequencing was used to characterize the molecular makeup of the fourteen potential bacterial isolates that were chosen based on their PGP traits, enzyme, and antagonistic activity. The length of the 16S rRNA gene sequence varies, and it is made up of both conserved and variable sections. On the basis of 16S rRNA gene sequencing, isolate PL2 showed maximum homology (98.81%) with *S. maltophilia* strain APP36. Isolate PL3 showed maximum homology (99.75%) with *M. thalassium* strain N40. Isolates PD3, PD5, SL1, SL2, JL1, JL2, JL4, and JD1 were characterized as belonging to different strains of *B. subtilis.* Isolate PD3 showed maximum homology (98.50%) with *B. subtilis* strain SCSRD3. Isolate PD5 showed maximum homology (99.88%) with *B. subtilis* strain MK720491.1. Isolate SL1 showed maximum homology (99.75%) with *B. subtilis* strain MO2. Isolate SL2 showed maximum homology (96.52%) with *B. subtilis* strain DV9-50. Isolate JL1 showed maximum homology (99.14%) with *B. subtilis* strain LSRBMoF-PIKRGCFTR17. Isolate JL2 showed maximum homology (100%) with *B. subtilis* strain HBUAS64159. Isolate JL4 showed maximum homology (100%) with *B. subtilis* strain A10. Isolate JD1 showed maximum homology (100%) with *B. subtilis* strain BSU3. Isolate JD2 showed maximum homology (99.69%) with *B. licheniformis* strain G1DM7. Isolate SL5 showed maximum homology (98.69%) with *A. gandavensis* strain IHBB 9448. Isolate SD3 and JL3 were characterized as belonging to different strains of *E. coli.* Isolate SD3 showed maximum homology (99.88%) with *E. coli* strain YZMc10-2. Isolate JL3 showed maximum homology (99.88%) with *E. coli* strain LCU-ID-EC4.

Nucleotide sequences have been submitted to the GenBank nucleotide sequence database, and accession no. has been obtained (OR573482-OR573489, OR739589-OR739593, OR826104). Seven strains of *B. subtilis*, two strains of *E. coli*, and each strain of *Bacillus* sp., *B. licheniformis*, *A. gandavensis*, *S. maltophilia*, and *M. thalassium* were identified and percent homology of 16S rRNA gene sequence of cow dung bacterial isolates with other nucleotide sequences present in the database using BLASTn analysis. Phylogenetic tree was also constructed using neighbor-joining tree method to find the distant relationship among the isolates as shown in Fig. 7.

Through 16S rRNA sequencing, it was found that *Bacillus* sp. was abundant in bacterial population of cow dung. Cow dung is a home to several bacteria including *E. coli*, *B. subtilis*, *M. thallassium*, *A. gandavensis*, and S. *maltophilia*. *E. coli* has been isolated from cow dung by several researchers [58–60]. *B. subtilis* has also been isolated from cow dung through different studies [61, 62]. Strains of *Microbacterium* sp. have been isolated from cow dung such as *M. suwonense* [63, 64], *M. stercoris* [65], *M. helvum* [66], and *M. bovistercoris* [67], although no reports on the isolation of *M. thalassium* from cow dung has been found. *A. gandavensis* has been isolated from the uterus of cattle [68]. *S. maltophilia* has been isolated from organic amendments and agricultural soils [69]. *B. licheniformis* has been isolated from cow manure [70].

Pot experiment on pea crop

Initial microbial count and chemical parameters of composite soil

Microbial analysis of composite soil was done on nutrient agar before starting the experiment and microbial count was found to be 85×10^6 cfu g^{-1}. Physical parameters of composite soil have been analyzed by using standard methods and the percent values of N, P, and K of composite soil was 210.86 kg ha^{-1}, 18.7 kg ha^{-1}, and 185 kg ha^{-1}, respectively.

Growth parameters

Perusal of data related to growth parameters as shown in Table 7 shown that maximum plant height was recorded in PL2 (14.76 cm) followed by JL2 (13.56 cm) which was significantly higher ($p < 0.05$) as compared to uninoculated control (10.02 cm). Minimum plant height was recorded in uninoculated control (10.02 cm) which was statistically at par ($p < 0.05$) with JL4 treatment (10.53 cm) and statistically ($p < 0.05$) different from other treatments. Germination percent was recorded highest in JL4, JL1, and control (91.77%) followed by JD1 (91.44%) which was statistically at par ($p < 0.05$) with each other. Minimum germination percent was recorded in case of treatment JD2 (57.89%) which was significantly different ($p < 0.05$) from other treatments. Minimum days to 1st flowering was found in JL4 and JD1 (48.33 days) followed by SD3 (49.67 days), JL1 (49.00 days), JL3 (49.33 days), and JD2 (48.67 days) which was statistically significant ($p < 0.05$) as compared to uninoculated control (54.67 days).

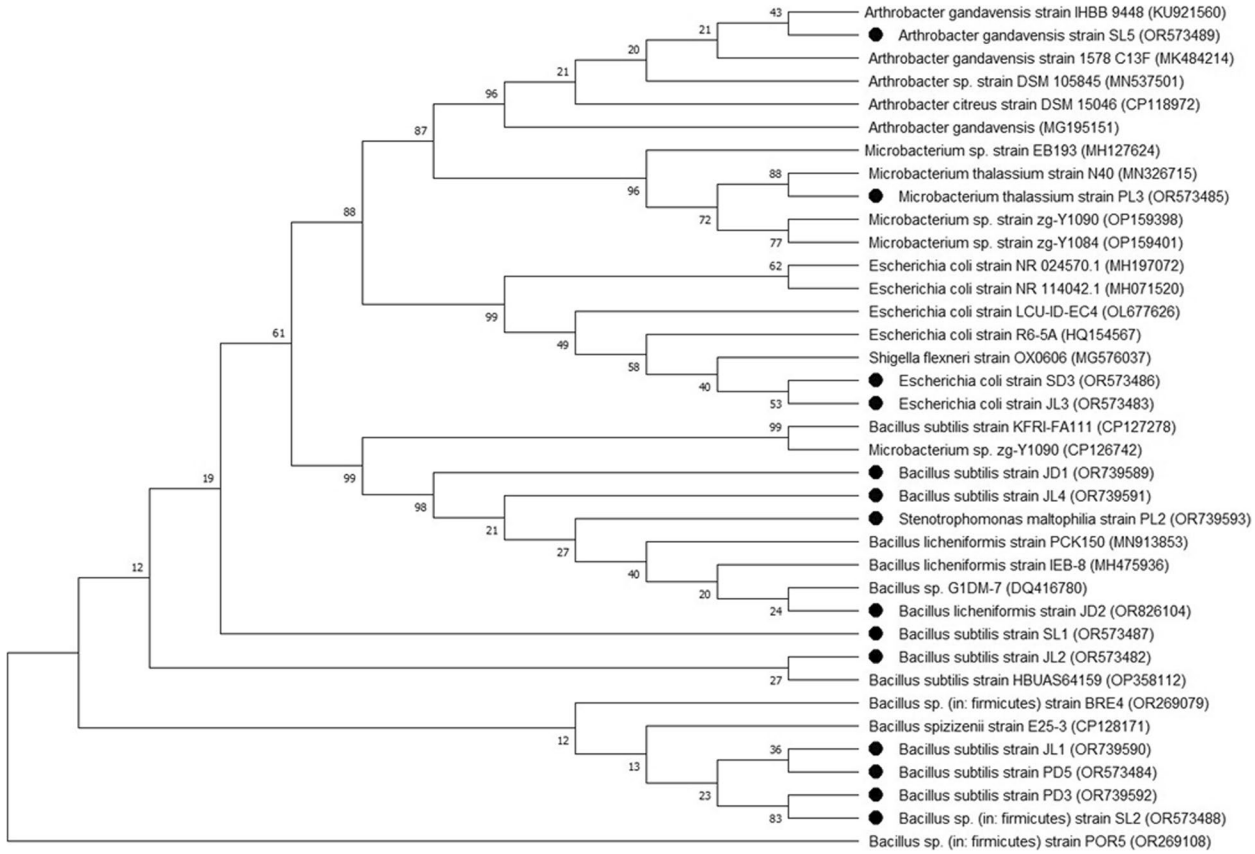


Fig. 7 Phylogenetic relationship of (•) selected bacterial isolates by using neighbor-joining tree method

Maximum days to 1st flowering was also found in uninoculated control. The pods number per plant was noted maximum in PD5 (3.33) followed by PL2 and JD2 (2.67) which was statistically significant ($p < 0.05$) as compared to uninoculated control and most of other treatments, whereas minimum pods per plant was recorded in SL1 (1.00) and SL5 (1.00). In case of uninoculated control, pods per plant was found to be 1.67.

The purpose of the pot experiment study was to determine whether cow dung isolates may improve soil quality and plant growth parameters. Most of the cow dung isolates significantly increased plant height, germination percent, pods per plant of pea seedlings and microbial count, and N, P, and K content of soil. Similar results were obtained in a pot culture experiment using cow dung bacteria to evaluate the growth promotion of *Capsicum annuum* L. [38]. Comparable findings were observed by Gohil et al. [71] when they added *Bacillus* sp. PG-8 culture to the *Arachis hypogea* plant, which significantly increased plant growth. Earlier reports also support such study, where plant growth-promoting bacterial treatment has resulted in increased nutritive value, along with enhanced plant growth and yield [72].

It is interesting to note that several cow dung isolates in this investigation were shown to drastically reduce the percentage of seeds germination and pods per plant compared to the untreated control, most likely by the production of volatile compounds [73]. Upon analysis, these harmful bacterial isolates failed to produce any HCN in vitro. Radha and Rao [45] also found that out of seven strains tested in the green house, only two strains of *Bacillus cereus* (PG2 and PG4) could increase the shoot length of maize significantly. Consequently, it is possible that additional gaseous metabolites the bacteria produced under these circumstances prevented the germination of the seeds. This observation is supported by the increase in percent seed germination by isolate JL1 and JL4 which otherwise did not produce HCN under in vitro conditions [74].

A few isolates may have a detrimental influence on growth because they produce particular metabolites that are harmful to the health of the plant. This demonstrates that some PGPRs, even those with high PGPR activity produced in vitro, have detrimental effects on plant growth and health. Thus, it is imperative that all PGPB be tested, if not up to net house conditions [74]. Under

Table 7 Plant growth parameters of pea crop under different treatments conducted in pot experiment

Treatments	Plant height (in cm)	Germination percent	Days to 1st flowering	Pods/plant
PL2	14.76	75.33	53.00	2.67
PL3	12.46	83.55	53.67	2.33
PD3	10.80	75.67	51.33	2.00
PD5	11.33	83.22	51.67	3.33
SL1	12.17	66.77	50.33	1.00
SL2	11.00	75.33	50.00	2.00
SL5	13.56	67.11	51.00	1.00
SD3	12.83	83.55	49.67	1.67
JL1	12.87	91.77	49.00	1.67
JL2	13.50	83.22	50.00	2.67
JL3	12.37	74.67	49.33	1.67
JL4	10.53	91.77	48.33	1.67
JD1	11.30	91.44	48.33	2.00
JD2	12.27	57.89	48.67	2.67
Control	10.02	91.77	54.67	1.67
SE(m)±	0.20	0.65	0.48	0.48
LSD ($p<0.05$)	0.59	1.88	1.39	1.39

SE (m) ± standard error mean

LSD least significant difference

PL Himachali Pahari lactating, *PD Himachali Pahari* non-lactating, *SL* Sahiwal lactating, *SD* Sahiwal non-lactating, *JL* Jersey lactating, *JD* Jersey non-lactating, *PB Pahari* bull

controlled circumstances, Dubeikovsky et al. [75] demonstrated that bacteria generating a high amount of IAA were harmful to sour cherries. Since the investigations were conducted in non-sterile soil, the variations in plant growth promotion among the isolates are attributable to their unique rhizospheric capabilities. The well-known and complex phenomena of bacterial plant growth promotion is frequently attained by the actions of multiple PGP traits displayed by bacteria associated with plants. Under in vitro applications, each of these isolates from cow dung shown a varying degree of PGP activities, including P-solubilization, IAA synthesis, and siderophore production.

Microbial count and chemical parameters

Maximum chemical properties, viz., N (313.60 kg ha^{-1}), P (40.31 kg ha^{-1}), and microbial count (4.7×10^8 cfu g^{-1}), were found in PL2 treatment, while K (253.74 kg ha^{-1}) was found to be maximum in PD3 which was statistically significant ($p<0.05$) as compared to uninoculated control and most of other treatments as shown in Table 8. Minimum microbial count (1.9×10^8 cfu g^{-1}), N (83.62 kg ha^{-1}), and P (15.79 kg ha^{-1}) was found in the control, whereas minimum K (202.10 kg ha^{-1}) was found in SL2. This might be due to better nutrient mobilization by bacteria, making it available in soil and thus their uptake by the leaves, roots, and fruits of host plant resulting in increment of seedling growth. Recent studies also provide evidence that application of PGPB results in increment of chemical parameters and microbial count in soil [76].

Conclusion

Cow dung of indigenous and exotic breeds contains beneficial microorganisms having various PGP traits, enzyme activity, and possessing antifungal activity. This provides the basis for preparing bioinoculants used for enhancing the plant health, disease management, and improving soil quality. Physicochemical parameters and microbial population was found maximum in *Himachali Pahari* cow dung which is good for maintaining plant health. PGP traits was shown by all cow dung isolates, irrespective of their breeds. Sahiwal and Jersey cow dung isolates found to be good for enzyme activity, whereas antifungal activity was shown maximum only by Jersey cow dung isolates. *M. thalassium* was first time reported from cow dung of *Himachali Pahari* and other strains showed similarity with *Bacillus* sp., *B. subtilis*, *B. licheniformis*, *E. coli*, *A. gandavensis*, and *S. maltophilia*. Pot experiment studies

Table 8 Microbial count and chemical parameters of pea crop under different treatments conducted in pot experiment

Treatments	Microbial count ($\times 10^8$ cfu g^{-1})	N (kg ha^{-1})	P (kg ha^{-1})	K (kg ha^{-1})
PL2	4.7	313.60	40.31	224.55
PL3	3.4	250.88	22.33	215.57
PD3	3.9	188.16	31.34	253.74
PD5	3.7	156.80	35.89	233.53
SL1	4.0	156.80	26.92	211.08
SL2	3.1	125.44	26.95	202.10
SL5	2.9	250.88	35.89	233.53
SD3	3.9	125.44	22.33	211.80
JL1	2.8	94.08	29.08	220.06
JL2	4.1	156.80	17.95	233.53
JL3	2.1	125.44	26.92	220.06
JL4	4.6	156.80	35.89	233.53
JD1	3.4	94.08	33.67	224.55
JD2	2.6	125.44	29.08	226.80
Control	1.9	83.62	15.79	224.55
SE(m)±	0.06	2.70	0.06	0.01
LSD ($p<0.05$)	0.18	7.83	0.16	0.03

SE (m) ± standard error mean

LSD least significant difference

PL Himachali Pahari lactating, *PD Himachali Pahari* non-lactating, *SL* Sahiwal lactating, SD Sahiwal non-lactating, *JL* Jersey lactating, *JD* Jersey non-lactating, *PB Pahari* bull

concludes that isolate PL2 (*S. maltophilia*), PD5 (*B. subtilis*), JL1 (*Bacillus subtilis*), JL4 (*Bacillus subtilis*), and JD1(*Bacillus subtilis*) was found to be best for growth parameters, microbial count, and N, P, and K parameters as compared to other nine isolates used for pot experiment studies. These potential five strains isolated from dung of *Himachali Pahari and* Jersey lactating and non-lactating cow breeds can be used singly or in consortium depend upon their antagonism or synergism property to protect, enrich, and flourish the soil microbial community as well as plant and soil health in future applications for field studies.

Supplementary Information

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Additional file 1: Table S1. Morphological characters of the bacteria isolated from cow dung of different breeds.

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Authors' contributions
In order to recognize the authors'participation, we highlight each individual contribution: Abhishek Walia and Rameshwar Kumar designed the study and supervised the experiments, Shweta Sagar, Jyoti Bala and Arjun Singh contributed in performing the experiment and writing of the manuscript. Rakesh Chauhan and Anila Badiyal reviewed and edit the manuscript. All these authors have substantial contributions to the final manuscript and approved this submission.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
The authors consent to the publication of this manuscript.

Competing interests
The authors declare no competing interests.

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