ORIGINAL ARTICLE

Smokeless Tobacco Harbors Bacteria Involved in Biofilm Formation as Well as Salt and Heavy Metal Tolerance Activity

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Abstract

In our previous culture-independent study on smokeless tobacco products, we have observed a strong positive correlation between several bacteria and genes involved in nitrate/nitrite reduction, biofilm formation, and pro-inflammation. Therefore, the present investigation was carried out to analyze the inhabitant bacterial population of the Indian ST products for assessing the health-associated risk attributes using culture-dependent approach. Traditional cultivation approaches recovered several bacterial isolates from commercial ST products on different culture media. A high colony formation unit (CFU) count was observed that ranged from 173×10^4 to 630.4×10^5 per gram of ST products. Of the 74 randomly selected and distinct bacterial isolates, 17 isolates showed a significantly enhanced growth (p-value < 0.05) in the presence of the aqueous tobacco extract. On biochemical characterization, these bacteria were identified as the member of *Bacillus, Enterobacter, Micrococcus, Providencia, Serratia, Pantoea, Proteus,* and *Pseudomonas*. Most of these bacteria also exhibited biofilm-forming activity, where eight bacterial isolates were identified for strong biofilm-forming action. 16S rRNA-based molecular characterization of these bacteria identified them as *Bacillus subtilis, Bacillus paralicheniformis, Enterobacter* sp*., Serratia marcescens, Pantoea anthophila,* and *Enterobacter cloacae*. Moreover, these bacteria also exhibited the potential to withstand high salt and heavy metal concentrations. The findings demonstrate that Indian ST products are heavily populated with wide bacterial species exhibiting potential in biofilm formation, heavy metal resistance, and salt tolerance.

Keywords Smokeless tobacco · Bacterial assessment · Biofilm formation · Oral cavity · Health risks · India

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Introduction

Smokeless tobacco (ST) is the non-combustible tobacco form used by people of almost every inhabited land on earth (National Cancer Institute and Centers for Disease Control and Prevention, 2014). It accounts for approximately 300 million ST users worldwide [1]. In a recent study, tobacco consumption was attributed to 8.71 million deaths and 229.77 Disability-adjusted life years (DALYs) worldwide [2]. Asian and other developing countries bear the major burden of ST users where 82% of ST users exist [3]. Due to high diversity, smokeless tobacco is considered a group of products where huge differences occur at the level of composition, processing, storage, and spatiotemporal conditions [1, 4]. Thus, assessment of the indigenous ST products at the local level must be considered to incorporate effective in-house policies. Smokeless tobacco serves as a reservoir of more than 400 toxicants and carcinogens [5]. Though remarkable development has been done in tobacco chemistry, however, the information on microorganisms in and on surfaces of ST products and their role is in the developing phase. In the last few years, some progress has been achieved in the line of exploring bacterial diversity and functional attributes of ST products [6–8]. Tobacco-inhabitant bacteria contribute a significant role due to their dynamic metabolism [9]. For example, nitrite accumulation in tobacco leaves is the consequence of bacterial metabolic activity where nitrate reductase converts available nitrates into nitrites [10–12]. The accumulated nitrites may further participate in tobacco-specific nitrosamines (TSNAs) formation by reacting with tobacco alkaloids either by dissimilatory or periplasmic nitrate reduction pathways [13]. Besides, acetaldehyde-producing bacteria (*Rothia*, *Streptococcus*, *Neisseria*, and *Corynebacterium*) have also been reported as bacterial constituents of various ST products [14–16]. Serval harmful moieties such as lipopolysaccharides (LPS), bacterial toxins, pro-inflammatory molecules, and fragmented peptidoglycans have been also reported from ST-inhabitant bacteria. In our previous investigation, we reported the taxonomic and functional profiling of bacteria of indigenous smokeless tobacco products using a 16S rRNA-based metagenomic approach [8]. This previous study comes out with several key findings and predicted many bacterial-derived risks to human health due to tobacco consumption. A strong positive correlation was also observed between bacteria and genes involved in nitrate/nitrite reduction, biofilm formation, and pro-inflammation [8]. Therefore, the culture-independent approach paved the way to explore the culturable bacterial communities in a rational way to understand the bacterial-derived risk attributes. In the present investigation, we conceived to explore the inhabitant bacteria from indigenous tobacco products for various risk attributes such as biofilm formation activity, salt tolerance and heavy metal tolerance.

Materials and Methods

Sample Collection and Physicochemical Properties of ST Products

Five commercial ST products were collected from the local market of Lucknow city of Uttar Pradesh, India. The samples were stored in sterile zip bags at 4 °C until their processing. Various physicochemical parameters were studied from each tobacco sample.

Determination of Color and Texture

The colour and texture were determined phenotypically. The pH of the sample was determined by suspending 1.0 gm of dried tobacco leaves in 10 ml sterile MilliQ water [17]. The samples were vortexed for 10 min followed by centrifugation at 10,000 rpm. The supernatant was used to determine the pH using a pH meter (Shimadzu, Japan) over a period of 10 min to achieve the stable pH readings.

Particle Size and Moisture Content Determination

Tobacco particle size was recorded for each sample by measuring the length and width to the nearest millimetre of more than 20 particles to calculate the standard deviation. The moisture content of the ST leaves was determined by the oven drying method [18]. Briefly, 5.0 gm of tobacco leaves were weighed and dried for 2–3 h at 104 °C to achieve the constant weight of the sample. The relative moisture content (%) of the product was measured by calculating the difference between the initial and final weight of the sample.

Determination of Nitrate and Nitrite Concentration

Nitrate concentration in tobacco extract was determined by the spectrophotometric screening method (APHA, 2005). Absorbance was recorded at two different ODs viz., 220 nm and 275 nm. Finally, the nitrate concentration was calculated by considering the absorbance at 275 nm and at 220 nm as follows. As dissolve organic matter shows absorption at 220 nm as well as 275 nm, while nitrate exhibits absorption only at 220 nm. Thus, the nitrate concentration was calculated by subtracting the absorbance at 275 nm from the absorbance at 220 nm to calculate the final absorbance of nitrate as follows. Similarly, nitrite concentration was also determined by the spectrophotometric method described by Narayana and Sunil (2009) [19] by recording the absorbance of the samples at 493 nm. Standard curves were prepared by taking varying concentrations of nitrate (10–1000 μg/ml) and nitrite (10–1000 μg/ml).

Determination of Nicotine Concentration in Tobacco Extract

The concentration of nicotine in tobacco samples was measured by analyzing the chromatogram of standard nicotine as described in Tassew and Chandravanshi (2015) [20]. Nicotine (Sigma–Aldrich, USA) with 99.9% purity was used as standard. A stock solution of 10 mM of nicotine was prepared in high-pressure liquid chromatography (HPLC grade water followed by serial dilution to achieve the different concentrations (0.1, 0.3, 0.5, 1.0, and 2.0) mM for the preparation of the calibration curve. Sample preparation was done by weighing 0.5 gm of oven-dried powdered tobacco leaves that were suspended in 10 ml of 25 mM phosphate buffer (pH 7.8) in a water bath adjusted at 30 °C for 24 h with continuous stirring. The aqueous extract was filtered through a 0.45 μM disc filter (Whatman™ Puradisc 25 mm) and stored in the refrigerator (4 °C) until its use in high-pressure liquid chromatography (HPLC). The isocratic mobile phase was prepared with solution A [100:99:1 (v/v/v):: acetonitrile: water: formic acid] and solution B (1:1 (v/v):: methanol: acetonitrile). The flow rate of 2.0 ml/min was used. Further, the mobile phase was

filtered through a 0.45 μM cellulose disc filter (Whatman™ *Puradisc* 25 mm). The filtered sample was injected into the HPLC column and the nicotine was detected by using a UV–VIS detector aided in HPLC (Model: LC-20AP, SHIMADZU, Japan) at 259 nm wavelength. The calibration curve of nicotine was derived from the peak area of standard solutions range 0.1 to 2 mM. The level of nicotine ranged between 17–27 mg in the tobacco samples studied here (Table 1).

Determination of Bacterial Load and Isolation of ST Inhabitant Bacteria

1.0 gm of each ST sample was crushed well and suspended in 10 ml of sterile phosphate buffer saline (PBS) solution (0.1 M; pH 8.0) for isolating the bacteria. The suspended solution was appropriately vortexed and appropriately diluted samples were spread on different culture media (Nutrient agar, MacConkey agar, EMB agar, Tryptic Soy agar, and Sheep Blood agar) to capture the maximum bacterial diversity. The respective Petri dishes were incubated at 37 °C for varying time intervals in an incubator. The bacterial load from each sample was measured by calculating the colony forming unit (CFU) and distinct colonies were picked randomly from each Petri dish. The colonies were purified and studied for further investigation. Glycerol stocks were made of pure isolates by suspending their respective culture broth in 40% (v/v) glycerol solution in a 1:1 ratio and storing them at -80 °C.

Preparation of Tobacco Aqueous Extract and its Effect on Bacterial Growth

The tobacco extract was prepared according to the protocol described by Liu et al. (2016) [21]. Briefly, 2.0 gm of oven-dried and powdered ST sample was soaked in sterile Milli Q water followed by intermittent sonication for 30 min with 30 s on/off mode. The suspension was centrifuged at 10,000 rpm for 10 min to obtain the tobacco aqueous extract. The supernatant was further filtered through a 0.22 μM syringe filter (Whatman™ Puradisc 25 mm). The effect of tobacco extract was observed on the growth of tobacco bacterial isolates in varying concentrations (1, 5, 10, 20, and 30) mg/ml in the nutrient broth.

Determination of Biofilm Formation Activity

Bacteria that showed elevated growth or were not inhibited in the presence of tobacco extract were further assessed for biofilm formation activity. Biofilm activity was performed through the tissue culture plate method [22] as well as Congo red agar method [23]. For quantitative biofilm formation activity, the bacterial isolates from a fresh agar plate were inoculated in the trypticase soy (TS) broth with 1% (w/v) glucose for overnight at 37 °C. The overnight grown culture was diluted in a 1:100 ratio in fresh TS medium. 200 μL of this broth was transferred into sterile 96-well polystyrene tissue culture-treated plates. *Staphylococcus aureus* was used as a positive control, whereas for the negative control, the sterile broth was used in the same culture plate. The plate was incubated at 37 °C for 24 h to develop the biofilm. The broth from each well was removed by a gentle tapping after the incubation. The wells were washed with 200 μL of sterile 0.1 M PBS (pH 7.0) buffer to remove the floating bacteria. Biofilm forming wall adhering bacteria were fixed by 2% (w/v) sodium acetate followed by staining with crystal-violet (0.1%) solution. The plates were dried after multiple washing with sterile distilled water. The biofilm formation activity was measured using an ELISA auto reader (Molecular Devices Spectra Max ABS Plus,

CA, USA) by measuring optical density at 540 nm. The entire experiment was carried out in triplicates to calculate the standard deviation.

Whereas for the qualitative estimation, the bacterial isolates were measured using the Congo red method [23]. The bacterial isolates were cultivated on autoclaved Congo red agar (CRA) medium. The medium was prepared by using 3.7% (w/v) Brain Heart Infusion broth (BHI) medium supplemented with 5% (w/v) sucrose, 5% (w/v) agar, and 0.8% (w/v) Congo red solution. The isolates were cultivated aerobically for 24 h at 37 °C. The isolates having black color crystalline consistency were identified as biofilm-forming bacteria.

Determination of Salt Tolerant Activity

An attempt was made to determine the effect of NaCl on selected bacterial isolates. For this, the bacterial growth was measured in the presence of a varying concentration of NaCl (0.5, 1.0, and 2.0) molar. The overnight-grown bacterial isolates were inoculated with 2% (v/v) culture broth in fresh nutrient broth. The effect of salt was recorded by measuring the optical density at 620 nm of the bacteria at varying time intervals using a spectrophotometer. The control of each isolate was designed in a similar manner in the absence of salt.

Determination of Screening of Heavy Metal Resistance Activity

Heavy metal resistance was determined by cultivating the selected isolates in the presence of different concentrations. Eight heavy metals Pb^{2+} (100–3000 ppm), Cr^{2+} (100–1500 ppm), Ni^{2+} (100–600 ppm), Fe^{3+} (100–1200 ppm), Cd^{3+} (100–3000 ppm), As^{2+} (100–500 ppm) and Hg^{2+} (50–100 ppm) and Sb^{3+} (50–200 ppm) were used in varying concentrations in nutrient agar media. The bacteria were grown at 37 °C overnight. Heavy metal resistance was determined by observing the appearance of growth of the isolates on respective metal ions under different concentrations as compared to the control i.e., on NA medium without metals. The minimum inhibitory concentration (MIC) was calculated by recording the minimum concentration of each metal where bacteria showed complete inhibition in its visible growth on Petri dishes.

Effect of Nicotine on Selected Isolates

A stock solution of nicotine (10 mM; Sigma–Aldrich, purity > 99.9%) was prepared in sterile Milli Q water. The effect of varying concentrations (0.5, 1.0, and 2.0) mM of nicotine was studied on selected bacterial isolates. The overnight grew bacterial isolates were inoculated with 2% (v/v) culture broth in a fresh nutrient broth medium. The effect of nicotine was recorded by measuring the optical density at 620 nm using a spectrophotometer. The control of each isolate was designed in a similar manner in the absence of nicotine.

Biochemical and Molecular Characterization of the Bacterial Isolates

Bacteria that showed elevated growth in the presence of tobacco extracts were characterized using biochemical methods and identified using Bergey's manual [24]. Whereas 16S-based molecular characterization was performed for only those bacterial isolates that showed strong biofilm formation. For this, the genomic DNA of respective bacterial isolates was extracted using the modified protocol of Verma and Satyanarayana

[25]. Briefly, 3.0 ml of the overnight grown culture of respective isolates were centrifuged at 10,000 rpm for 5 min to collect the bacteria pellet. The sedimented pellet was suspended in 1.0 ml extraction buffer (N-,N-,N-,N-cetyltrimethylammonium bromide 1%, polyvinylpolypyrrolidone (PVPP) 2%, 1.5 M NaCl, 100 mM EDTA, 100 mM TE buffer (pH 8.0), 100 mM sodium phosphate buffer (pH 8.0), and 100 μL RNase A; [25]). 2.5 μL each of lysozyme (10 mg/ml) and proteinase-K (10 mg/ml) were further added into the suspension and kept at 37 °C for 1 h followed by the addition of 50 μL of 10% SDS (w/v) solution. The bacterial pellet was allowed to lyse for another 1 h at 60 °C. The soup having lysed bacterial cells was treated with an equal volume of phenol: chloroform: iso-amyl alcohol (25:24:1) solution. The aqueous phase was collected after centrifugation (10,000 rpm) and treated with 0.7 V of isopropanol to precipitate the genomic DNA at room temperature for 1 h. The DNA pellet was collected after centrifugation at 10,000 rpm for 15 min at room temperature and washed with 70% (v/v) ethanol. The washed pellet was dried at room temperature and suspended in 50 μL of 0.1 M TE buffer (pH 8.0).

Sequence Analysis and Phylogenetic Tree Construction

Approximately 50 ng of respective genomic DNA was used as a template for amplifying full-length genes of bacterial-specific 16S rRNA region. The polymerase chain reaction (PCR) was carried out by using 16S rRNA primers (EUBF: *AGAGTTTGATCMTGGCTC AG* and EUBR: *AAGGAGGTGATCCANCCRCA*) (Lyons et al., 2005 [26]) in a 2×PCR master mix (GeneDireX, OnePCR™ *Plus*). The PCR reaction was set as initial denaturation at 95 °C followed by 29 cycles of denaturation (1 min at 95 °C), annealing (30 s at 56 °C), and extension (1 min at 72 °C). A final extension of 10 min was given at 72 °C. The amplicons were visualized at 1% (w/v) agarose gel and sequenced using the same set of primers at Barcode Biosciences, Bangalore, India. The sequence analysis of the amplicons (~1500 bp) was performed using BLASTn of NCBI.

Results

Physicochemical Properties of ST Products

The color and texture of the ST samples were greenish-yellow to brown having pH in the range of 5.2 to 8.6. Most of the samples (TS-1, TS-2, and TS-3) showed a pH in the acidic range, whereas TS-4 and TS-5 were alkaline (Table 1). The particle size was categorized as described by Han and co-workers [17]. The particle size of most of the samples was in the range of 3–10 mm, where TS-1, TS-2, and TS-5 exhibited larger sizes in the range of 8–10 mm. TS-3 and TS-4 were quite larger in size (Table 1). Moisture content was significantly varied among the samples, where TS-1 and TS-2 showed a low moisture content of 6.24% and 5.25% respectively. The maximum moisture content (48.1%) was identified in TS-4 followed by TS-5 (28.7%). The nitrate concentration was maximum in TS-4 (590.3 μg/g) followed by TS-2 and TS-3 (Table 1). Whereas, nitrite concentration was recorded as quite similar (~19 μg/g of ST) among the different samples studied here (Table 1).

Table 1 Various physicochemical properties of the ST products

Sample code	Category	Leaf appearance (phenotype)	pH	Moisture (%)	Nicotine content (mg)	Nitrate (µg/g)	Nitrite (µg/g)	Particle size (mm)
TS-1	Chewable tobacco	Greenish yellow	5.3 ± 0.15	6.24 ± 0.02	27.54 ± 0.46	343.3 ± 0.2	19.22 ± 0.16	8–10
TS-2	Chewable tobacco	Greenish yellow	5.1 ± 0.05	5.25 ± 0.15	17.5 ± 0.23	395.6 ± 0.17	19.32 ± 0.04	7–10
TS-3	Snus	Greenish yellow	5.5 ± 0.12	8.46 ± 0.07	19.44 ± 0.14	373.6 ± 0.08	19.33 ± 0.3	5–8
TS-4	Moist snuff	Brownish	8.6 ± 0.3	48.1 ± 0.2	17.82 ± 0.09	590.3 ± 0.15	19.14 ± 0.18	3–6
TS-5	Chewable tobacco	Greenish yellow	7.7 ± 0.1	28.7 ± 0.16	27.54 ± 0.2	326.6 ± 0.05	19.35 ± 0.21	8–10

ST Inhabitant Bacteria and Bacterial Load

Though the tobacco samples were purchased in the same season, even the bacterial load was significantly varied among tobacco samples. Overall, the average maximum bacterial load was obtained in TS-4 (630.4×10^4) followed by TS-5 (407.2×10^5), whereas the least CFU count was observed in TS-2 (173×10^4). While comparing media, nutrient agar media showed the maximum bacterial count (1925×10^5) followed by EMB agar medium (Table 2). Trypticase soy agar media revealed the minimum bacterial count. A total of 74 distinct bacterial isolates were observed that were phenotypically different from each other (Table 2). These isolates were picked and screened for their growth on tobacco extracts for further investigation.

Effect of Tobacco Aqueous Extract on Bacterial Growth

Of the 74 bacterial isolates, 17 isolates showed significantly enhanced growth ($p\text{-value} < 0.05$) in the presence of aqueous tobacco extract (Table 3). After 24 h of incubation, the isolate TS-3TSA-2 showed the maximum growth followed by TS4-NA2. Whereas, TS-3NA-4 exhibited the minimum but significantly enhanced growth. On biochemical characterization of these bacteria, five were identified as *Bacillus* spp., three *Enterobacter* spp., three *Micrococcus* spp., one each of *Siccibacter* sp., *Providencia* sp., *Serratia* sp., *Proteus* sp., and *Acinetobacter* sp. (Table 4). These bacterial isolates were further studied for biofilm formation activity.

Biofilm Formation Activity

Of the seventeen selected isolates, eight bacterial isolates (TS1-NA3, TS3-NA1, TS3-NA3, TS3-NA4, TS3-TSA, TS4-NA2, TS4-MA2, and TS5-MA2) exhibited strong biofilm formation activity (Fig. 1; Table 5). Of the remaining isolates, only one isolate (TS4-MA2) showed moderate (Fig. 1; Table 5) biofilm formation, while other six isolates (TS3-TSA3, TS4-NA1, TS4-NA7, TS4-MA3, TS4-TSA1, and TS4-EMB3) were weak in biofilm formation activity (Fig. 1; Table 5). The two isolates (TS1-EMB1 and TS5-TSA2) did not show any biofilm formation activity either by tissue culture plate method or Congo red agar method.

Salt Resistant Activity

TS1-NA3, TS3-NA4, TS4-NA2, TS3-TSA, and TS5-MA2 sustained their growth in the presence of 0.5 M NaCl. TS3-NA1 and TS3-NA3 slightly inhibited the growth in the presence of 0.5 M NaCl. Whereas at 1 M of NaCl, only isolate (TS3-NA4) showed an increase in its growth, and the remaining seven isolates (TS1-NA3, TS3-NA1, TS3-NA3, TS3-TSA, TS4-NA2, TS4-MA2, and TS5-MA2) were inhibited (Fig. 2A and 2B; Table 6).

Heavy Metal Resistant Activity

These bacterial isolates also showed metal tolerance activities against various heavy metals. The isolate TS4-NA2 exhibited maximum metal tolerance against Pb^{2+} and Cd^{2+}, up to 3000 ppm. It exhibited tolerance against Fe^{3+}, Cr^{3+}, Ni^{2+} and As^{3+}- 1200 ppm, 1500 ppm,

Table 2 Total CFU count of bacterial isolates obtained from different ST products

ST products	CFU/gm of ST product					Average CFU	No. of distinct isolates
	Nutrient agar	MacConkey agar	EMB agar	Trypticase soy agar	Sheep blood agar		
TS-1	112×10^4	268×10^4	228×10^4	380×10^4	400×10^4	277.6×10^4	14
TS-2	145×10^4	110×10^4	150×10^4	210×10^4	250×10^4	173×10^4	12
TS-3	468×10^4	168×10^4	176×10^4	568×10^4	416×10^4	359.2×10^4	13
TS-4	952×10^5	520×10^5	760×10^5	400×10^4	880×10^5	630.4×10^5	18
TS-5	248×10^5	104×10^5	720×10^5	484×10^5	480×10^5	407.2×10^5	17
Total colonies							**74**

Table 3 The optical density (OD) of bacterial isolates in the presence of smokeless tobacco extract (20 mg/ml)[#]

S. No	Bacterial isolates	Control (OD 600 nm) at 24 h	Test (OD 600 nm) at 24 h	p-value
1	TS-1 NA-1	0.482 ± 0.026	0.518 ± 0.013	0.154
2	TS-1 NA-2	0.395 ± 0.01	0.397 ± 0.008	0.887
3	TS-1 NA-3	0.493 ± 0.008	0.694 ± 0.014	0.018*
4	TS-1 NA-4	0.58 ± 0.013	0.576 ± 0.006	0.754
5	TS-1 SBA-1	0.252 ± 0.005	0.314 ± 0.01	0.525
6	TS-1 SBA-2	0.247 ± 0.008	0.293 ± 0.007	0.413
7	TS-1 SBA-3	0.224 ± 0.004	0.29 ± 0.012	0.382
8	TS-1 MA-1	0.416 ± 0.011	0.547 ± 0.008	0.103
9	TS-1 MA-2	0.467 ± 0.009	0.5 ± 0.011	0.605
10	TS-1 MA-3	0.228 ± 0.240	0.374 ± 0.383	0.105
11	TS-1 EMB-1	0.27 ± 0.008	0.503 ± 0.02	0.041*
12	TS-1 EMB-2	0.462 ± 0.011	0.495 ± 0.008	0.804
13	TS-1 TSA-1	0.376 ± 0.009	0.395 ± 0.036	0.511
14	TS-1 TSA-2	0.351 ± 0.006	0.392 ± 0.005	0.631
15	TS-2 NA-1	0.636 ± 0.007	0.692 ± 0.008	0.646
16	TS-2 NA-2	0.531 ± 0.011	0.621 ± 0.012	0.373
17	TS-2 NA-3	0.361 ± 0.014	0.46 ± 0.010	0.349
18	TS-2 NA-4	0.696 ± 0.900	0.738 ± 0.015	0.818
19	TS-2 NA-5	0.515 ± 0.01	0.597 ± 0.007	0.625
20	TS-2 EMB-1	0.387 ± 0.009	0.44 ± 0.011	0.459
21	TS-2 EMB-2	0.481 ± 0.008	0.547 ± 0.018	0.450
22	TS-2 EMB3	0.333 ± 0.007	0.34 ± 0.012	0.757
23	TS-2 SBA-1	0.361 ± 0.007	0.378 ± 0.600	0.595
24	TS-2 SBA-2	0.355 ± 0.008	0.434 ± 0.006	0.481
25	TS-2 TSA	0.245 ± 0.009	0.296 ± 0.007	0.118
26	TS-2 MA	0.482 ± 0.008	0.57 ± 0.009	0.453
27	TS-3 NA-1	0.155 ± 0.009	0.293 ± 0.01	0.035*
28	TS-3 NA-2	0.504 ± 0.008	0.589 ± 0.005	0.568
29	TS-3 NA-3	0.548 ± 0.028	0.968 ± 0.009	0.036*
30	TS-3 NA-4	0.557 ± 0.025	0.859 ± 0.013	0.046*
31	TS-3 NA-5	0.456 ± 0.035	0.406 ± 0.010	0.671
32	TS-3 EMB-1	0.546 ± 0.009	0.475 ± 0.006	0.983
33	TS-3 EMB-2	0.543 ± 0.006	0.612 ± 0.014	0.845
34	TS-3 SBA-1	0.452 ± 0.012	0.590 ± 0.009	0.141
35	TS-3 SBA-2	0.525 ± 0.010	0.564 ± 0.010	0.319
36	TS-3 TSA-1	0.471 ± 0.012	0.576 ± 0.009	0.373
37	TS-3 TSA-2	0.682 ± 0.009	0.897 ± 0.013	0.049*
38	TS-3 TSA-3	0.387 ± 0.01	0.679 ± 0.022	0.032*
39	TS-3 MA	0.477 ± 0.009	0.326 ± 0.011	0.073
40	TS-4 NA-1	0.263 ± 0.01	0.772 ± 0.005	0.001*
41	TS-4 NA-2	0.694 ± 0.007	1.026 ± 0.307	0.007*
42	TS-4 NA-3	0.515 ± 0.009	0.53 ± 0.008	0.727
43	TS-4 NA-4	0.332 ± 0.102	0.349 ± 0.124	0.786

Table 3 (continued)

S. No	Bacterial isolates	Control (OD 600 nm) at 24 h	Test (OD 600 nm) at 24 h	*p-value*
44	TS-4 NA-5	0.559 ± 0.007	0.684 ± 0.011	0.158
45	TS-4 NA-6	0.425 ± 0.010	0.527 ± 0.007	0.492
46	TS-4 NA-7	0.572 ± 0.009	1 ± 0.075	0.001*
47	TS-4 EMB-1	0.54 ± 0.005	0.6 ± 0.011	0.300
48	TS-4 EMB-2	0.798 ± 0.013	1.5 ± 0.01	0.046*
49	TS-4 SBA-1	1.01 ± 0.010	1.03 ± 0.008	0.743
50	TS-4 SBA-2	1.332 ± 0.068	1.349 ± 0.015	0.877
51	TS-4 SBA-3	1.76 ± 0.091	1 ± 0.015	0.955
52	TS-4 SBA-4	0.125 ± 0.011	0.233 ± 0.007	0.069
53	TS-4 TSA-1	0.665 ± 0.016	1 ± 0.001	0.018*
54	TS-4 TSA-2	0.482 ± 0.007	0.542 ± 0.008	0.398
55	TS-4 MA-1	0.83 ± 0.012	1.02 ± 0.022	0.140
56	TS-4 MA-2	0.587 ± 0.014	1.99 ± 0.171	0.033*
57	TS-4 MA-3	0.635 ± 0.02	0.99 ± 0.031	0.009*
58	TS-5 NA-1	0.472 ± 0.03	0.536 ± 0.005	0.371
59	TS-5 NA-2	0.225 ± 0.01	0.321 ± 0.008	0.095
60	TS-5 NA-3	0.251 ± 0.04	0.283 ± 0.02	0.849
61	TS-5 NA-4	0.237 ± 0.007	0.284 ± 0.01	0.537
62	TS-5 NA-5	0.268 ± 0.009	0.297 ± 0.006	0.577
63	TS-5 MA-1	0.29 ± 0.045	0.573 ± 0.003	0.030
64	TS-5 MA-2	0.29 ± 0.275	0.573 ± 0.447	0.030*
65	TS-5 MA-3	0.226 ± 0.02	0.354 ± 0.007	0.134
66	TS-5 SBA-1	0.3011 ± 0.01	0.371 ± 0.009	0.582
67	TS-5 SBA-2	0.255 ± 0.004	0.312 ± 0.051	0.289
68	TS-5 SBA-3	0.246 ± 0.009	0.276 ± 0.008	0.789
69	TS-5 SBA-4	0.348 ± 0.054	0.437 ± 0.021	0.248
70	TS-5 TSA-1	0.248 ± 0.007	0.276 ± 0.032	0.649
71	TS-5 TSA-2	0.294 ± 0.016	0.381 ± 0.042	0.040*
72	TS-5 EMB-1	0.238 ± 0.009	0.25 ± 0.017	0.717
73	TS-5 EMB-2	0.289 ± 0.006	0.388 ± 0.024	0.359
74	TS-5 EMB-3	0.377 ± 0.01	0.515 ± 0.021	0.050*

* Indicates significant *p-value* < 0.05

#: Of the varying concentration, 20 mg/ml concentration was selected to compare the growth due to maximum growth among the bacterial isolates

800 ppm and 500 ppm respectively. This isolate further showed tolerance against Hg^{2+} and Sb^{3+} at 100 ppm, and 200 ppm respectively. TS1-NA3 (Cr^{3+}-1500 ppm; Fe^{3+}- 1200 ppm; Cd^{2+}- 3000 ppm) and TS3-TSA (Pb^{2+}-1500 ppm; Ni^{2+}-600 ppm; Fe^{3+}- 1200 ppm) showed multi-metal tolerance activity. The two bacterial strains (TS3-NA1 and TS3-NA3) showed similar profiles by exhibiting maximum metal tolerance against Fe^{3+} and Ni^{2+} up to 1200 ppm and 600 ppm respectively. Whereas, TS3-NA3 was also tolerant against Hg^{2+} (50 ppm) and Sb^{3+} (100 ppm). Similarly, TS5-MA2 showed tolerance up to 1200 ppm and 3000 ppm respectively for Fe^{3+} and Cd^{2+}. Bacterial strain TS3-NA4 was

Table 4 Biochemical observations of the seventeen isolates based on tobacco extract experiment

Biochemical test	TS1-NA3	TS1-EMB1	TS3-NA1	TS3-NA3	TS3-NA4	TS3-TSA2	TS3-TSA3	TS4-NA1	TS4-NA2	TS4-NA7	TS4-MA2	TS4-MA3	TS4-TSA1	TS4-EMB2	TS5-EMB3	TS5-MA2	TS5-TSA2
Gram stain	(-)	(-)	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(-)	(+)	(-)	(-)
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Cocci	Rod	Cocci	Rod	Rod	Rod	Rod	Cocci	Rod	Rod
Oxidase	-	+	+	+	+	+	-	+	-	+	-	+	-	-	+	-	+
Catalase	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Motility	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(-)	(+)	(+)	(+)	(-)	(+)	(-)
Indole	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Methyl red	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)
Voges Proskauer	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(-)
Urease	(-)	(-)	(-)	(-)	+	(-)	(-)	(-)	+	(-)	+	(-)	(-)	+	(-)	(-)	(-)
Citrate test	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Amylase	(-)	(-)	(+)	(+)	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)
TSI (triple sugar iron)	(+)	(+)	(-)	(+)	(+)	(-)	(-)	(-)	H_2S	H_2S, Gas	(+)	(-)	(-)	(+) Gas	(-)	(+) Gas	(+)
Lactose	(+)	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(-)	(+)	(-)
Casein hydrolysis	(+)	(-)	(+)	(+)	(-)	(+)	(-)	(-)	(+)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(+)
Mannitol	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(-)	(+)	(+)	(-)	(-)	(+)	(-)
Probable identity	Siccibacter sp.	Providencia sp.	Bacillus sp.	Bacillus sp.	Bacillus sp.	Bacillus sp.	Enterobacter sp.	Micrococcus sp.	Serratia sp.	Micrococcus sp.	Pantoea sp.	Bacillus sp.	Enterobacter spp.	Proteus sp.	Micrococcus sp.	Enterobacter sp.	Acinetobacter sp.

(-): Negative, (+): Positive, Gas: Gas production, H_2S: Hydrogen sulfide production
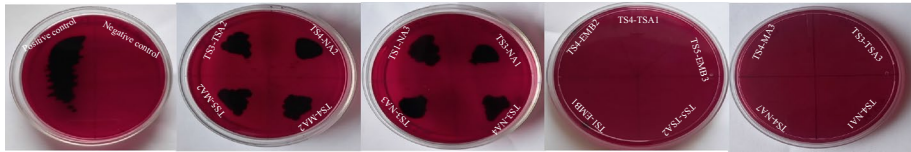
Fig. 1 Screening of bacterial isolates from ST products by congo red agar (CRA) method

Table 5 Screening of selected isolates for biofilm formation activity by tissue culture plate, and Congo-Red agar methods

S. No	Isolates	TCP method	CRA method	Biofilm formation activity*
1	TS1-NA3	1.47 ± 0.372	Black crystalline appearance	Strong biofilm former
2	TS1-EMB1	0.178 ± 0.0015	No black color appears	No biofilm former
3	TS3-NA1	0.833 ± 0.126	Black crystalline appearance	Strong biofilm former
4	TE3-NA3	1.431 ± 0.046	Black crystalline appearance	Strong biofilm former
5	TS3-NA4	3.637 ± 0.022	Black crystalline appearance	Strong biofilm former
6	TS3-TSA2	1.361 ± 0.320	Black crystalline appearance	Strong biofilm former
7	TS3-TSA3	0.264 ± 0.031	No black color appears	Weak biofilm former
8	TS4-NA1	0.292 ± 0.044	No black color appears	Weak biofilm former
9	TS4-NA2	2.343 ± 0.206	Black crystalline appearance	Strong biofilm former
10	TS4- NA7	0.214 ± 0.027	No black color appears	Weak biofilm former
11	TS4-MA2	3.473 ± 0.016	Black crystalline appearance	Strong biofilm former
12	TS4-MA3	0.197 ± 0.021	No black color appears	Weak biofilm former
13	TS4-TSA1	0.252 ± 0.021	No black color appears	Weak biofilm former
14	TS4-EMB2	0.711 ± 0.065	No black color appears	Weak biofilm former
15	TS5-EMB3	0.233 ± 0.040	No black color appears	Weak biofilm former
16	TS5-MA2	2.305 ± 0.132	Black crystalline appearance	Strong biofilm former
17	TS5-TSA2	0.176 ± 0.039	No black color appears	No biofilm former

*Interpretation of biofilm production

$OD_{cut} = OD_{avg}$ of negative control $+ 3 \times$ standard deviation of ODs of negative control

$OD \leq OD_{cut} =$ Non biofilm former

$OD_{cut} < OD \leq 2 \times OD_{cut} =$ Weak biofilm former

$2 \times O_{cut} < OD \leq 4 \times OD_{cut} =$ Moderate biofilm former

$OD > 4 \times OD_{cut}$ Strong biofilm former

able to tolerate heavy metal i.e., Ni^{2+} up to 600 ppm and Sb^{3+} up to 100 ppm. The strain TS4-MA2 was intolerant to various metals studied in this investigation (Table 6).

Effect of Nicotine on Selected Bacterial Isolates

Nicotine was not stimulatory to enhance the growth of any of these eight selected bacterial isolates. Four bacterial strains (TS1-NA3, TS4-NA2, TS4-MA2, and TS5-MA2) sustained their growth in presence of 0.5–1.0 mg/ml nicotine. Whereas, four strains (TS3-NA1, TS3-NA3, TS3-NA4, and TS3-TSA) were slightly inhibited at both concentrations of nicotine (Supplementary Fig. 1).
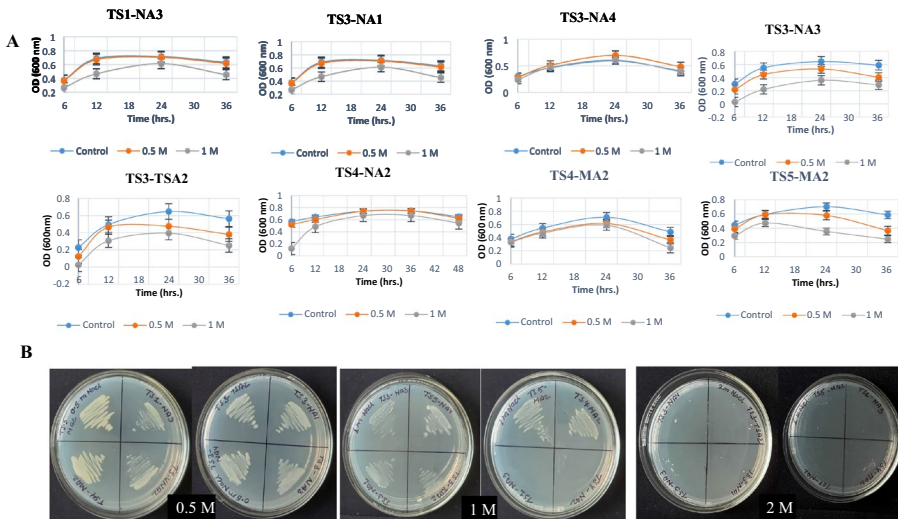
Fig. 2 Growth profile of bacterial isolates at varying concentrations of NaCl (**A**) in nutrient broth and on nutrient agar (**B**)

Molecular Identification of the Selected Isolates

All eight bacterial isolates showed the desired amplification of 1480 bp of bacterial-specific 16S rRNA gene using bacterial-specific 16S primers. On sequencing, the four isolates (TS3-NA1, TS3-NA3, TS3-NA4, and TS3-TSA2) were identified as members of the phylum Firmicutes. Of them, three isolates (TS3-NA1, TS3-NA3, and TS3-TSA2) showed maximum identity with *Bacillus subtilis*, whereas TE3-NA4 was identified as *Bacillus paralicheniformis*. The remaining other four isolates were members of Pseudomonadota. These isolates were identified as *Serratia marcescens* (TS4-NA2), *Pantoea anthophila* (TS4-MA2), *Enterobacter cloacae* (TS5-MA3), and Uncultured *Enterobacter* sp. (TS1-NA3) (Table 7).

Discussion

To date, only two reports are available that discuss the bacterial diversity of smokeless tobacco products using the traditional cultivation approach [17, 27]. Han and colleagues paved the way to explore the bacterial diversity of ST products to protect public health and develop new policies for its effective cessation [17]. The investigation carried out by Smyth and co-workers discusses the CFU count of the respective samples only [4]. However, several metagenomic-based analyses have further predicted the presence of pathogenic and opportunistic pathogens among various ST products [6, 8, 28, 29]. Due to the major burden (> 85%) on South and southeast countries of ST consumers and the share of affected deaths and DALYs (Siddiqi et al., 2020 [1]), it compels us to understand the bacterial diversity of ST products. Only one report discusses the bacterial diversity of Indian smokeless tobacco products using biochemical analysis of bacterial isolates [27]. The study uncovered the dominance of *Pseudomonas aeruginosa* and *Streptococcus faecalis* followed by a

Table 6 Minimum inhibitory concentration (MIC) of NaCl and different heavy metals on selected bacterial isolates

NaCl conc. (mol/l)	Bacterial isolates							
	TS1-NA3	TS3-NA1	TS3-NA3	TS3-NA4	TS3-TSA2	TS4-NA2	TS4-MA2	TS5-MA2
0.5	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)
1.0	(-)	(+)	(+)	(-)	(+)	(+)	(+)	(-)
2.0	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)

Heavy metal (ppm)	Bacterial isolates							
	TS1-NA3	TS3-NA1	TS3-NA3	TS3-NA4	TS3-TSA2	TS4-NA2	TS4-MA2	TS5-MA2
$Pb(NO_3)_2$	(-)	(-)	(-)	(-)	1500	3000	(-)	(-)
$K_2Cr_2O_7$	1500	(-)	(-)	(-)	(-)	1500	(-)	(-)
$NiCl_2$	(-)	600	600	600	600	800	(-)	(-)
$FeCl_3$	1200	1200	1200	(-)	1200	1200	(-)	1200
$CdCO_3$	3000	(-)	(-)	(-)	(-)	3000	(-)	3000
Na_3AsO_2	(-)	(-)	(-)	(-)	(-)	500	(-)	(-)
$HgCl_2$	(-)	(-)	50	(-)	(-)	100	(-)	(-)
$K_2Sb_2C_8H_4O_{12} \cdot 3H_2O$	(-)	(-)	100	100	(-)	200	(-)	(-)

(+): Growth appeared; (-): No Growth

Table 7 BLASTn analysis of 16S rRNA sequence of selected bacterial isolates

Bacterial isolates (NCBI accession number)	Maximum identity with- (Top three)	Identity (%)	NCBI accession number
TS1-NA3 (OQ194120)	*Siccibacter colletis*	98.90%	JF357616.1
	Salmonella enterica	98.90%	CP002433.1
	Pantoea sp.	98.90%	FJ930077.1
TS3-NA1 (OP854924)	*Bacillus subtilis*	99.79%	KX495304.1
	Bacillus subtilis	99.66%	OM807213.1
	Bacillus halotolerance	99.73%	KJ787122.1
TS3-NA3 (OP854923)	*Bacillus subtilis*	97.85%	OM807213.1
	Bacillus subtilis	97.98%	EF032678.1
	Bacillus subtilis	97.91%	KX495304.1
TS3-NA4 (OP854925)	*Bacillus paralicheniformis*	97.04%	MK063857.1
	Bacillus paralicheniformis	97.04%	MK063845.1
	Bacillus paralicheniformis	97.04%	MW301648.1
TS3-TSA2 (OP854931)	*Bacillus subtilis*	99.38%	JX502843.1
	Bacillus mojavensis	99.38%	HM753629.1
	Bacillus sp.	99.38%	KY283146.1
TS4-NA2 (OP854932)	*Serratia marcescens*	99.86%	FJ360759.1
	Serratia marcescens	99.86%	CP053286.1
	Serratia marcescens	99.86%	CP050013.1
TS4-MA2 (OP854933)	*Pantoea anthophila*	99.86%	CP110473.1
	Pantoea anthophila	99.86%	JN644500.1
	Pantoea sp. Sc1	99.86%	AY924374.1
TS5-MA2 (OP854934)	*Enterobacter cloacae*	97.29%	MK575033.1
	Enterobacter sp.	97.29%	MK575030.1
	Enterobacter sp.	97.29%	MK575027.1

non-significant share of *Klebsiella* spp., *E. coli*, and *Bacillus subtilis* among Indian ST products. However, the study relies on biochemical characterization of the isolates which needs further confirmation by using molecular-level (16S rRNA) identification. Moreover, the study was limited to the taxonomy assignment level only and lacks any functional characterization to identify the health risks. Therefore, extensive research is required to explore the bacterial diversity of Indian ST products to uncover the bacterial-associated risk attributes. In the present investigation, bacterial diversity was done on five different Indian commercial ST products. The bacterial load ranges from 630.4×10^4 to 407.2×10^5 per gram of ST products among various samples and it shows a heavy load of bacterial biomass on Indian ST products which may contribute in altering one's oral bacteriome. Han et al. (2016) also reported a high bacterial load on various tobacco products such as moist snuff (1.05×10^6 CFU/g STP), snus (8.33×10^1 CFU/g STP), and chewing tobacco products (average of 2.54×10^5 CFU/g STP) [17]. A viable elevated count of 9.37×10^7 CFU was also observed in toombak leaves from various tobacco products [4]. The altered oral microbiome has been reported for the onset of several oral diseases [30]. Tobacco metabolites have been reported to perturb the oral bacterial diversity and are among the major causes of oral cancers [8, 10]. Nicotine and like compounds react with nitrite to form carcinogenic

TSNA compounds [13]. In this investigation, a consistent level of nitrite was observed among various tobacco products (Table 1). Nitrite formation is bacterial derived transformation of nitrate which react with nicotine and their derivatives to synthesize various forms of TSNAs [10, 12, 13]. In a recent investigation, chewing smokeless tobacco showed significant alteration in the oral bacteriome of healthy individuals [9]. *Fusobacteria, Porphyromonas-, Desulfobulbus-, Enterococcus-,* and *Parvimonas*-like genera were significantly higher in abundance in the oral cavity of tobacco chewers. Moreover, *Lactobacillus* and *Haemophilus* were depleted in the ST chewers' oral cavity where most of them also show potential in biofilm formation. A similar finding was observed during the metagenomic analysis of Indian ST products that showed a strong correlation with the genes involved in biofilm formation of *E. coli*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* type [8]. Moreover, cigarette tobacco extracts (CSEs) have also been identified as a stimulator for enhancing growth as well as biofilm formation activity [31]. A significant elevation (p-value < 0.05) in the growth profile of seventeen bacterial isolates was observed in the presence of aqueous tobacco extracts here. Liu et al. (2016) observed that smokeless tobacco extract (STE) shows a concentration-dependent effect on growth and biofilm formation activity on oral pathogens [21]. Bagaitkar et al. (2011) reported that tobacco extracts induce biofilm formation by over-expressing *FimA* gene and reducing the capsular polysaccharides of *Porphyromonas gingivalis* [32]. These isolates were characterized as the members of *Bacillus, Enterobacter, Micrococcus, Providencia, Serratia, Pantoea, Proteus,* and *Pseudomonas.* None of these bacteria have ever been investigated for studying growth effects in the presence of STEs. Moreover, this is a preliminary information drawing that needs to be further confirmed by considering a larger number of tobacco-inhabitant bacteria. The report of Liu et al. (2016) [21]) and Dubois et al. (2014) [33]) discusses the effect of STEs on oral pathogens only [21, 33]. Interestingly, the isolates showing elevated growth in the presence of STEs also exhibited biofilm formation activity (Table 4). Thus, it would be interesting to study the correlation between tobacco-specific compounds and their effect on biofilm formation either *in-silico* or by experimentation. Pure nicotine has been reported for exhibiting a stimulatory effect on growth and biofilm formation activity on several oral pathogens [21, 34, 35]. Huang and colleagues [36] reported that nicotine induces the upregulation of many binding proteins involved in biofilm formation. For example, metal ABC transporter substrate-binding lipoproteins, Streptococcal surface protein (A and B), Amylase-binding protein (AbpA and AbpB), glucosyltransferase G and, surface-associated protein (CshA and CshB) assist in binding proteins during biofilm formation. However, we did not observe nicotine has a stimulatory effect on the growth of ST-derived biofilm-forming bacteria here. *Salmonella enterica*, *Pantoea anthophila*, *Serratia marcescens*, and *Enterobacter cloacae* were however able to sustain their growth up to 1.0 mg/ml of nicotine. Whereas, all *Bacillus* spp. here exhibited an inhibitory effect at any concentration of nicotine on their growth. Therefore, besides nicotine, other prevalent chemical compounds of ST products must be carefully screened for their role in the biofilm-formation activity. Here, the biofilm formation activity was studied among only those isolates that showed elevated growth in the presence of STEs. However, their biofilm activity was assessed in the absence of any tobacco compound or tobacco extracts. On molecular identification, these bacteria were identified as various strains of *Bacillus subtilis*, *Bacillus paralicheniformis*, *Serratia marcescens*, *Pantoea anthophila*, *Enterobacter cloacae*, and one uncultured bacterium showing close identity with *Salmonella enterica*. *Bacillus* spp. have already been reported as one of the major genera of tobacco products [17, 37, 38]. Moreover, the identified *Bacillus* spp. has a potential role in biofilm formation. For example, *Bacillus subtilis*; a Gram-positive, soil-dwelling, non-pathogenic, model biofilm

former produces an extracellular matrix composed of products of multiple operons such as *epsA-O* [39–42]. In a recent investigation, the co-culturing of *Bacillus subtilis* and *Pantoea agglomerans* showed more protective biofilm where both share the structural elements [43]. Therefore, the dynamics of *Bacillus-Pantoea* biofilm formation must be studied in the presence of tobacco extracts. The presence of *Serratia marcescens* must be carefully examined for presence in ST products. A significant count of this bacterium (an *Enterobacter*) was observed during the investigation. It has emerged as a nosocomial opportunistic pathogen that naturally dwells in soil, water, and plants [44, 45]. *Enterobacter cloacae* have been observed as another nosocomial pathogen in ST products that causes a wide variety of infections such as urinary tract infections, pneumonia, and septicemia [46, 47]. The species has grabbed attention in recent years due to its role in spreading carbapenem-resistant infections [48, 49]. In an early investigation, *Enterobacter cloacae* were reported as a nicotine degrader in tobacco plants [50] and cause stem rot disease in tobacco [51]. Bacterial biofilms are well known for exhibiting the potential for protecting bacteria cells under adverse environmental conditions such as exposure to heavy metals, toxic chemicals, salt, and pH [52–54]. Similar properties for exhibiting tolerance towards salt (NaCl) and heavy metals (Cr^{3+}, Pb^{2+}, Cu^{2+}, Cd^{2+}, As^{3+}, Ni^{2+}, Hg^{2+}, and Sb^{2+}) among biofilm-forming bacteria were observed in the present investigation. Thus, it is a more frightening condition for chewing ST products, where several such bacteria introduce into the oral cavity while chewing tobacco. *B. subtilis*, *Bacillus* spp., and *B. paralicheniformis* have been reported for high salt-tolerant activity [17, 55]. We also observed *B. paralicheniformis* as the most salt-tolerant bacterium among the studied isolates here. Several strains of *Bacillus subtilis* have also been reported for heavy metal resistance characteristics [56–58]. However, *B. paralicheniformis* have not yet been reported for heavy metal resistance and need to be characterized for understanding the mechanisms involved. Another biofilm-forming bacterium, *Serratia marcecens*, also withstands high salt and metal-tolerant activity. *Serratia marcecens* strains have been reported for high salt-tolerant activity [59, 60]. Whereas, Sahoo and co-workers reported *Serratia* sp. GP01 is a multi-metal-resistant bacteria from a fertilizer plant [61]. Similarly, *Enterobacter cloacae* strains have also been reported for high salt-tolerant [62, 63] and multi-metal [64] activity. *Pantoea* spp. also investigated for heavy metal-resistant potential [65, 66] and salt-resistant potential [67, 68]. Wei-Xie et al. [69] reported a *Pantoea* sp. PP4 exhibits biosorption capability for Pb and bioprecipitation for Pb and Cd [22]. The occurrence of such bacteria indicates heavy metal contamination among ST products that may be due to either over-mineralization of tobacco plants during cultivation or processing of the tobacco in industries.

Conclusion

The high bacterial load, tobacco-stimulated growth of the bacterial isolates, and their biofilm-forming characteristic make the ST products nastier to consume in any form. Besides, tobacco-inhabitant bacteria further showed tolerance towards high concentrations of heavy metals and salts. Therefore, chewing tobacco act as carrier of health risk-associated bacteria into the oral cavity and may assist proliferation of several oral diseases. Microbiological facet of smokeless tobacco however needs extensive research to explore the dynamics of bacteria. The present findings warn to assess the tobacco-associated bacterial population for various health-associated risk factors including the antibiotic resistance profile of inhabitant bacteria of ST products. With these findings, a scientific basis for sincere

concern about making cessation policies on ST products are provided. Especially, in South and South-East Asian countries, where ST product consumers predominate as compared to the other parts and oral hygiene is almost ignored. It should also be noted that India harbors the second largest consumer and tobacco producer.

Author's Contribution DV conceived the idea and designed the experiments. AV performed the experimental work. AV and DV wrote and edited the manuscript. Both authors read and approved the final version of the manuscript.

Data Availability My manuscript has no associated data.

Declarations

Ethics Approval There is no participation of human and animal, and their biological materials, therefore no ethics approval is required.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

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References

1. Siddiqi, K., Husain, S., Vidyasagaran, A., Readshaw, A., Mishu, M. P., & Sheikh, A. (2020). Global burden of disease due to smokeless tobacco consumption in adults: An updated analysis of data from 127 countries. *BMC Medicine, 18*, 1–22.
2. He, H., Pan, Z., Wu, J., Hu, C., Bai, L., & Lyu, J. (2022). Health effects of tobacco at the global, regional, and national levels: results from the 2019 global burden of disease study. *Nicotine & Tobacco Research, 24*, 864–870.
3. Yang, H., Ma, C., Zhao, M., Magnussen, C. G., & Xi, B. (2022). Prevalence and trend of smokeless tobacco use and its associated factors among adolescents aged 12–16 years in 138 countries/territories, 1999–2019. *BMC Medicine, 20*, 1–2.
4. Smyth, E. M., Kulkarni, P., Claye, E., Stanfill, S., Tyx, R., Maddox, C., Mongodin, E. F., & Sapkota, A. R. (2017). Smokeless tobacco products harbor diverse bacterial microbiota that differs across products and brands. *Applied Microbiology and Biotechnology, 101*, 5391–5403.
5. Vishwakarma, A., & Verma, D. (2020). Exploring the microbiome of smokeless tobacco. In R. Chaudhary & A. Verma (Eds.), *Microorganisms for sustainable environment and health* (pp. 167–178). Elsevier.
6. Tyx, R. E., Stanfill, S. B., Keong, L. M., Rivera, A. J., Satten, G. A., & Watson, C. H. (2016). Characterization of bacterial communities in selected smokeless tobacco products using 16S rDNA analysis. *PLoS One, 11*, e0146939.
7. Sajid, M., Sharma, P., Srivastava, S., Hariprasad, R., Singh, H., & Bharadwaj, M. (2022). Smokeless tobacco consumption induces dysbiosis of oral mycobiome: A pilot study. *Applied Microbiology and Biotechnology, 106*, 5643–5657.

8. Vishwakarma, A., Srivastava, A., Mishra, S., & Verma, D. (2023). Taxonomic and functional profiling of Indian smokeless tobacco bacteriome uncovers several bacterial-derived risks to human health. *World Journal of Microbiology & Biotechnology, 39*, 1–17.
9. Srivastava, S., Sajid, M., Singh, H., & Bharadwaj, M. (2022). Delineating the bacteriome of packaged and loose smokeless tobacco products available in North India. *Applied Microbiology and Biotechnology, 106*, 4129–4144.
10. Law, A. D., Fisher, C., Jack, A., & Moe, L. A. (2016). Tobacco, microbes, and carcinogens: Correlation between tobacco cure conditions, tobacco-specific nitrosamine content, and cured leaf microbial community. *Microbial Ecology, 72*, 120–129.
11. Di Giacomo, M., Paolino, M., Silvestro, D., Vigliotta, G., Imperi, F., & Visca, P. (2007). Microbial community structure and dynamics of dark fire-cured tobacco fermentation. *Applied and Environment Microbiology, 73*, 825.
12. Fisher, M. T., Bennett, C. B., Hayes, A., Kargalioglu, Y., Knox, B. L., Xu, D., Muhammad-Kah, R., & Gaworski, C. L. (2012). Sources of and technical approaches for the abatement of tobacco specific nitrosamine formation in moist smokeless tobacco products. *Food and Chemical Toxicology, 50*, 942–948.
13. Gonzalez, P. J., Correia, C., Moura, I., Brondino, C. D., & Moura, J. J. (2006). Bacterial nitrate reductases: molecular and biological aspects of nitrate reduction. *Journal of Inorganic Biochemistry, 100*, 1015–1023.
14. Mason, M. R., Preshaw, P. M., Nagaraja, H. N., Dabdoub, S. M., Rahman, A., & Kumar, P. S. (2015). The subgingival microbiome of clinically healthy current and never smokers. *ISME Journal, 9*, 268–272.
15. Halboub, E., Al-Ak'hali, M. S., Alamir, A. H., Homeida, H. E., Baraniya, D., Chen, T., & Al-Hebshi, N. N. (2020). Tongue microbiome of smokeless tobacco users. *BMC Microbiology, 8*, 201.
16. Vishwakarma, A., & Verma, D. (2021). Microorganisms: Crucial players of smokeless tobacco for several health attributes. *Applied Microbiology and Biotechnology, 105*, 6123–6132.
17. Han, Q.-Q., Lü, X.-P., Bai, J.-P., Qiao, Y., Paré, P. W., Wang, S.-M., Zhang, J.-L., Wu, Y.-N., Pang, X.-P., Xu, W.-B., & Wang, Z.-L. (2014). Beneficial soil bacterium *Bacillus subtilis* (GB03) augments salt tolerance of white clover. *Frontiers in Plant Science, 5*, 525.
18. Jin, X., Shi, C., Yu, C. Y., Yamada, T., & Sacks, E. J. (2017). Determination of leaf water content by visible and near-infrared spectrometry and multivariate calibration in Miscanthus. *Frontiers in Plant Science, 8*, 721.
19. Narayana, B., & Sunil, K. (2009). A spectrophotometric method for the determination of nitrite and nitrate. *EJAC, 4*, 204–214.
20. Tassew, Z., & Chandravanshi, B. S. (2015). Levels of nicotine in Ethiopian tobacco leaves. *Springer Plus, 4*, 1–6.
21. Liu, M., Jin, J., Pan, H., Feng, J., Cerniglia, C. E., Yang, M., & Chen, H. (2016). Effect of smokeless tobacco products on human oral bacteria growth and viability. *Anaerobe, 42*, 152–161.
22. Christensen, G. D., Simpson, W. A., Younger, J. J., Baddour, L. M., Barrett, F. F., Melton, D. M., & Beachey, E. H. (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: A quantitative model for the adherence of staphylococci to medical devices. *Journal of Clinical Microbiology, 22*, 996–1006.
23. Freeman, J., Falkiner, F. R., & Keane, C. T. (1998). New method for detecting slime production by coagulase negative *Staphylococci. Journal of Clinical Pathology, 42*, 872–874.
24. Bergey, D. H., & Holt, J. G. (2000). Bergey's manual of determinative bacteriology. 9th ed. Philadelphia, Lippincott Williams & Wilkins
25. Verma, D., & Satyanarayana, T. (2011). An improved protocol for DNA extraction from alkaline soil and sediment samples for constructing metagenomic libraries. *Applied Biochemistry and Biotechnology, 165*, 454–464.
26. Lyons, I. J., Newell, Y. S., Brown, P. R., & Moran, A. M. (2005). Screening for bacterial–fungal associations in a south-eastern US salt marsh using pre-established fungal monocultures, FEMS Microbiol. *Ecol., 54*, 179–187.
27. Mehra, R., Mohanty, V., Balappanavar, A.Y., & Kapoor, S. (2020). Bacterial contamination of packaged smokeless tobacco sold in India. *Tobacco Prevention & Cessation, 6*, 11.
28. Sajid, M., Srivastava, S., Kumar, A., Kumar, A., Singh, H., & Bharadwaj, M. (2021). Bacteriome of moist smokeless tobacco products consumed in India with emphasis on the predictive functional potential. *Frontiers in Microbiology, 12*, 784841
29. Sajid, M., Srivastava, S., Joshi, L., & Bharadwaj, M. (2021). Impact of smokeless tobacco-associated bacteriome in oral carcinogenesis. *Anaerobe, 70*, 1–9.
30. Verma, D., Garg, P. K., & Dubey, A. K. (2018). Insights into the human oral microbiome. *Archives of Microbiology, 200*, 525–540.

31. Goldstein-Daruech, N., Cope, E. K., Zhao, K. Q., Vukovic, K., Kofonow, J. M., Doghramji, L., González, B., Chiu, A. G., Kennedy, D. W., Palmer, J. N., & Leid, J. G. (2011). Tobacco smoke mediated induction of sinonasal microbial biofilms. *PLoS One, 6*, e15700.

32. Bagaitkar, J., Daep, C. A., Patel, C. K., Renaud, D. E., Demuth, D. R., & Scott, D. A. (2011). Tobacco smoke augments *Porphyromonas gingivalis-Streptococcus gordonii* biofilm formation. *PLoS One, 6*, e27386.

33. DuBois, A. E., Bennett, Z. C., Khalid, U., Khalid, A., Meece, R. A., Difore, G. J., & Gregory, R. L. (2014). Nicotine: Its stimulating and inhibitory effects on oral microorganisms. *Fine Foc, 1*, 63–75.

34. Falkler, W. A., Jr., Zimmerman, M. L., Martin, S. A., & Hall, E. R. (1987). The effect of smokeless-tobacco extracts on the growth of oral bacteria of the genus *Streptococcus*. *Archives of Oral Biology, 32*, 221–223.

35. Vellappally, S., Fiala, Z., Smejkalova, J., Jacob, V., & Shriharsha, P. (2007). Influence of tobacco use in dental caries development. *Central European Journal of Public Health, 15*, 116–121.

36. Huang, R., Li, M., & Gregory, R. L. (2012). Effect of nicotine on growth and metabolism of Streptococcus mutans. *European Journal of Oral Sciences, 120*, 319–325.

37. Rubinstein, I., & Pedersen, G. W. (2002). *Bacillus* species are present in chewing tobacco sold in the United States and evoke plasma exudation from the oral mucosa. *Clinical and Vaccine Immunology, 9*, 1057–1060.

38. Srivastav, M., Dineshkumar, T., Priyadharini, S., Niveditha, T., Sk, P., & Rajkumar, K. (2020). Smoke-less tobacco products (STPs) harbour bacterial populations with potential for oral carcinogenicity. *Asian Pacific Journal of Cancer Prevention, 21*, 815–824.

39. Branda, S. S., Gonzalez-Pastor, J. E., Ben-Yehuda, S., Losick, R., & Kolter, R. (2001). Fruiting body formation by *Bacillus subtilis*. *Proceedings of the National academy of Sciences of the United States of America, 98*, 11621–11626.

40. Romero, D., Aguilar, C., Losick, R., & Kolter, R. (2010). Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms. *Proceedings of the National academy of Sciences of the United States of America, 107*, 2230–2234.

41. Kobayashi, K., & Iwano, M. (2012). BslA (YuaB) forms a hydrophobic layer on the surface of *Bacillus subtilis* biofilms. *Molecular Microbiology, 85*, 51–66.

42. Arnaouteli, S., Bamford, N. C., Stanley-Wall, N. R., & Kovács, Á. T. (2021). *Bacillus subtilis* biofilm formation and social interactions. *Nature Reviews Microbiology, 19*, 600–614.

43. Yannarell, S. M., Grandchamp, G. M., Chen, S. Y., Daniels, K. E., & Shank, E. A. (2019). A Dual-Species Biofilm with Emergent Mechanical and Protective Properties. *Journal of Bacteriology, 201*, e00670-e718.

44. Ghaith, D. M., Zafer, M. M., Ismail, D. K., Al-Agamy, M. H., Bohol, M. F. F., Al-Qahtani, A., Al-Ahdal, M. N., Elnagdy, S. M., & Mostafa, I. Y. (2018). First reported nosocomial outbreak of *Serratia marcescens* harboring *blaIMP-4* and *blaVIM-2* in a neonatal intensive care unit in Cairo. *Egypt. Infect. Drug Resist., 11*, 2211.

45. Ferreira, R. L., Rezende, G. S., Damas, M. S. F., Oliveira-Silva, M., Pitondo-Silva, A., Brito, M. C., Leon-ardecz, E., Góes, F. R. D., Campanini, E. B., Malavazi, I., & da Cunha, A. F. (2020). Characterization of KPC-producing *Serratia marcescens* in an intensive care unit of a Brazilian tertiary hospital. *Frontiers in Microbiology, 11*, 956.

46. Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P., & Edmond, M. B. (2004). Noso-comial bloodstream infections in US Hospitals: Analysis of 24,179 Cases from a Prospective Nationwide Surveillance Study. *Clinical Infectious Diseases, 39*, 309–317.

47. Annavajhala, M. K., Gomez-Simmonds, A., & Uhlemann, A. C. (2019). Multidrug-resistant *Enterobacter cloacae* complex emerging as a global, diversifying threat. *Frontiers in Microbiology, 10*, 44.

48. Wilson, B. M., El Chakhtoura, N. G., Patel, S., Saade, E., Donskey, C. J., Bonomo, R. A., & Perez, F. (2017). Carbapenem-resistant enterobacter cloacae in patients from the US veterans health administration, 2006–2015. *Emerging Infectious Diseases, 23*, 878–880.

49. Hu, H., Liu, Y., Huang, Y., Zhang, Z., & Tang, H. (2022). The Leaf Microbiome of Tobacco Plants across Eight Chinese Provinces. *Microorganisms, 10*, 450.

50. Ruiz, G. V. (1983). Nicotine degradation by bacteria *Enterobacter cloacae* as nicotine degrader. *Tobacco, 22*, 85–98.

51. Zhang, Y. C., Zhang, J. H., Shi, M. L., Wang, H. C., & Guo, Y. S. (2018). First report of enterobacter cloacae causing stem rot on tobacco in China. *Vegetos, 31*, 1.

52. Vu, B., Chen, M., Crawford, R. J., & Ivanova, E. P. (2009). Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules, 14*, 2535–2554.

53. Edwards, S. J., & Kjellerup, B. V. (2013). Applications of biofilms in bioremediation and biotransformation of persistent organic pollutants, pharmaceuticals/personal care products, and heavy metals. *Applied Microbiology and Biotechnology, 97*, 9909–9921.

54. Haque, M. M., Niloy, N. M., Khirul, M. A., Alam, M. F., & Tareq, S. M. (2021). Appraisal of probabilistic human health risks of heavy metals in vegetables from industrial, non-industrial and arsenic contaminated areas of Bangladesh. *Heliyon, 7*, ne06309.

55. Ibarra-Villarreal, A. L., Gándara-Ledezma, A., Godoy-Flores, A. D., Herrera-Sepúlveda, A., Díaz-Rod-ríguez, A. M., Parra-Cota, F. I., & de los Santos-Villalobos, S. (2021). Salt-tolerant Bacillus species as a promising strategy to mitigate the salinity stress in wheat (Triticum turgidum subsp. durum). *Journal of Arid Environments, 186*, 104399.

56. Bai, J., Yang, X., Du, R., Chen, Y., Wang, S., & Qiu, R. (2014). Biosorption mechanisms involved in immobilization of soil Pb by *Bacillus subtilis* DBM in a multi-metal-contaminated soil. *Journal of Environmental Sciences, 26*, 2056–2064.

57. Cai, Y., Li, X., Liu, D., Xu, C., Ai, Y., Sun, X., Zhang, M., Gao, Y., Zhang, Y., Yang, T., & Wang, J. (2018). Novel Pb-resistant *Bacillus subtilis* bacterium isolate for Co-biosorption of hazardous Sb(III) and Pb(II): thermodynamics and application strategy. *International Journal of Environmental Research and Public Health, 15*, 702.

58. Sizentcov, A., Sal'nikova, E., Barysheva, E., Sizentcov, Y., & Sal'nikova, V. (2020). Biotoxicity of heavy metal salts to *Bacillus subtilis* and their sorption properties. *E3S Web of Conferences, 157*, 02012.

59. Ketola, T., & Hiltunen, T. (2014). Rapid evolutionary adaptation to elevated salt concentrations in pathogenic freshwater bacteria *Serratia marcescens*. *Ecology and Evolution, 4*, 3901–3908.

60 Singh, R. P., & Jha, P. N. (2016). The multifarious PGPR serratia marcescens CDP-13 augments induced systemic resistance and enhanced salinity tolerance of wheat (triticum aestivum L.). *PLoS One, 11*, e0155026.

61. Sahoo, H., Kumari, S., & Naik, U.C. (2021). Characterization of multi-metal-resistant Serratia sp. GP01 for treatment of effluent from fertilizer industries. *Archives of Microbiology, 203*, 5425–5435.

62. Bhise, K. K., Bhagwat, P. K., & Dandge, P. B. (2017). Plant growth-promoting characteristics of salt tolerant *Enterobacter cloacae* strain KBPD and its efficacy in amelioration of salt stress in *Vigna radiata L. Journal of Plant Growth Regulation, 36*, 215–226.

63. Ji, C., Liu, Z., Hao, L., Song, X., Wang, C., Liu, Y., Li, H., Li, C., Gao, Q., & Liu, X. (2020). Effects of *Enterobacter cloacae* HG-1 on the nitrogen-fixing community structure of wheat rhizosphere soil and on salt tolerance. *Frontiers in Plant Science, 11*, 1094.

64. Kang, C. H., & So, J. S. (2016). Heavy metal and antibiotic resistance of ureolytic bacteria and their immobilization of heavy metals. *Ecological Engineering, 97*, 304–312.

65. Li, S., Wang, J., Gao, N., Liu, L., & Chen, Y. (2017). The effects of *Pantoea* sp. strain Y4–4 on alfalfa in the remediation of heavy-metal-contaminated soil, and auxiliary impacts of plant residues on the remediation of saline–alkali soils. *Canadian Journal of Microbiology, 63*, 278–286.

66. Mohite, B. V., Koli, S. H., & Patil, S. V. (2018). Heavy metal stress and its consequences on exopolysaccharide (EPS)-producing *Pantoea agglomerans*. *Applied Biochemistry and Biotechnology, 186*, 199–216.

67. Panwar, M., Tewari, R., Gulati, A., & Nayyar, H. (2016). Indigenous salt-tolerant *rhizobacterium Pantoea dispersa* (PSB3) reduces sodium uptake and mitigates the effects of salt stress on growth and yield of chickpea. *Acta Physiologiae Plantarum, 38*, 1–12.

68. Bhise, K. K., & Dandge, B. P. (2019). Alleviation of salinity stress in rice plant by encapsulated salt tolerant plant growth promoting bacteria *Pantoea agglomerans* strain KL and its root colonization ability. *Archives of Agronomy and Soil Science, 65*, 1955–1968.

69. WeiXie, L., Yang, R., Liu, B., Lei, N., Peng, S., Li, J., Tong, J., Deng, R., & Li, J. (2022). Effects of Pb-, Cd-resistant bacteriumP antoea sp. on growth, heavy metal uptake and bacterial communities in oligotrophic growth substrates of Lolium multiflorum Lam. *Environmental Science and Pollution Research, 29*, 50742–50754

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