Culture-dependent microbial profiling of Indian smokeless tobacco products

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Abstract:

In India, wide range of SLT products are available using variable production processes and additives. Present study characterize cultivable microbial diversity among eighteen commercially available Indian SLT products. Through conventional culture-based approaches 1776 cultivable aerobic, anaerobic, fungal, and nitrate/nitrite-reducing colonies were reported, out of which thirty-five bacterial isolates were further characterized as nitrate-reducing using biochemical identification assays. Antibiotic susceptibility testing was done to evaluate any potential health risks associated. Pan Masala samples exhibited the highest microbial diversity, followed by chewing tobacco leaves, whereas Khaini predominantly supported anaerobic populations. Zarda samples demonstrated comparatively low microbial loads, potentially due to higher chemical additives. The heterogeneous product group displayed variable fungal and nitrate-reducing communities but lacked detectable anaerobic populations. Among the nitrate-reducing isolates, none demonstrated pathogenic characteristics or multidrug resistance. Five strains exhibited enhanced nitrite-reduction capacity, indicating potential to reduce nitrite pool generation and hence possibly impacting the nitrosation processes linked to TSNA formation. Biochemical characterization suggested all nitrite-reducing isolates belongs to Enterobactereaceae group though, further characterization is required. Present study is first to systematically demonstrate the presence of metabolically active Nitrate/Nitrite-reducers in Indian SLT products. Collectively, the findings highlight pronounced microbiological variability across Indian SLT products and confirmed presence of nitrate and nitrite-reducing microbes. This study provides foundational evidence linking variable product characteristics with microbial ecology in SLTs.

Keywords: Smokeless tobacco, Tobacco-specific nitrosamines, Nitrate/Nitrite-reducers, Pan Masala, Khaini, Zarda, chewing tobacco, Gudaku, Kiwam

1. Introduction:

Smokeless tobacco products (SLTs) is an umbrella term used to represent heterogenous and diverse group of tobacco preparations consumed without combustion [1, 2]. South East Asian region housing 80% of global smokeless tobacco users has emerged as a major hub of tobacco related health issues [3-5]. India being the second largest consumer and producer of tobacco, has a variety of SLT products available at very low cost [6, 7]. SLT consumption have deep socio-cultural acceptance and tobacco have been consumed for centuries as different forms either alone or with beetle leaeves, areca nuts, spices and other ingadients [8]. SLTs are widely used through chewing as Pan masala, placing tobacco based content in the oral cavity as Khaini, applied as toothpaste, incorporated into edible preparations as Qiwam or through nasal administration such as naswar. Many SLT products are locally manufactured using traditional formulations as Dohra and Gudaku, while products such as Zarda, Pan Masal are often produced in small-scale industries with limited

regulatory supervision, potentially increasing the carcinogenecityof the final consumable SLT product [1].

Recent trends indicate a decline in cigarette smoking prevalence due to increased awareness, media campaigning, stricter regulations and social disapproval which has shifted many users towards smokeless tobacco alternatives. Owing to their social acceptability, affordability, easy availability and the widespread perception that SLT products are less harmful than smoked tobacco, SLTs has become the most prevalent form of tobacco consumption in South-East Asia particularly in India and neighbouring countries. India accounts for over 267 million (29% of all adults) SLT consumers acording to the Global Adult Tobacco Survey India, 2016-17 (GATS-2) and 1.35 million deaths every year due to tobacco use as per WHO report [7, 5].

Tobacco consumption remains one of the largest preventable risk factors contributing substentially to non-communicable disease burden, morbidity, mortality and healthcare expenditure in India [3, 9]. Extensive epidemiological studies has established strong association between SLT and cancers of the oral cavity, pharynx, numerous other types of cancers along with innumerable health complications as cardiovascular diseases and several other chronic diseases (Figure 1). A significant proportion of oral cancer-related deaths in India are directly attributed to smokeless tobacco consumption [10-14].

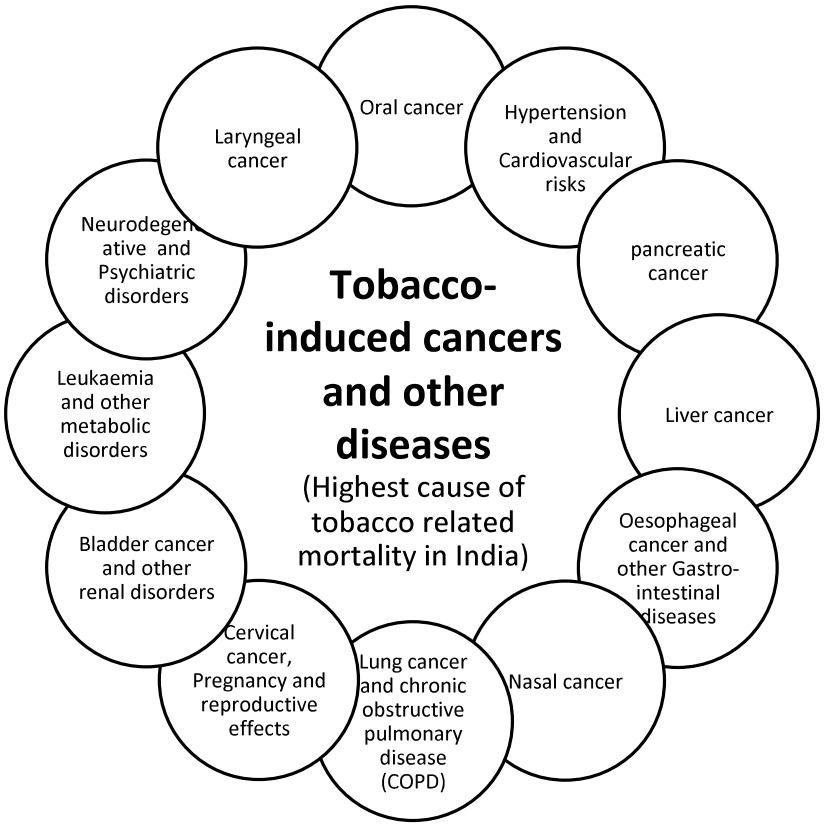

Fig 1: Health impacts of Indian ready-to-use Smokeless Tobacco Products

Although, Nicotine in tobacco products is percieved as primary harmful component of tobacco, it mainly contributes to tobacco addiction and is not strongly carcinogenic. The major carcinogenic agents in tobacco products are tobacco-specific nitrosamines (TSNAs), including N′-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosoanabasine (NAB), and N′-nitrosoanatabine (NAT). Fresh tobacco leaves contain negligible

levels of TSNAs but high concentrations of alkaloids such as nicotine, nornicotine, anabasine, anatabine etc. To reduce the bitter taste of alkaloids and to improve flavor, texture, and consumer acceptability, tobacco leaves undergo curing and fermentation, before product preparation. These post-harvest processes promote microbial and chemical nitrosation reactions that convert tobacco alkaloids into TSNAs (Figure 2) [15-17].

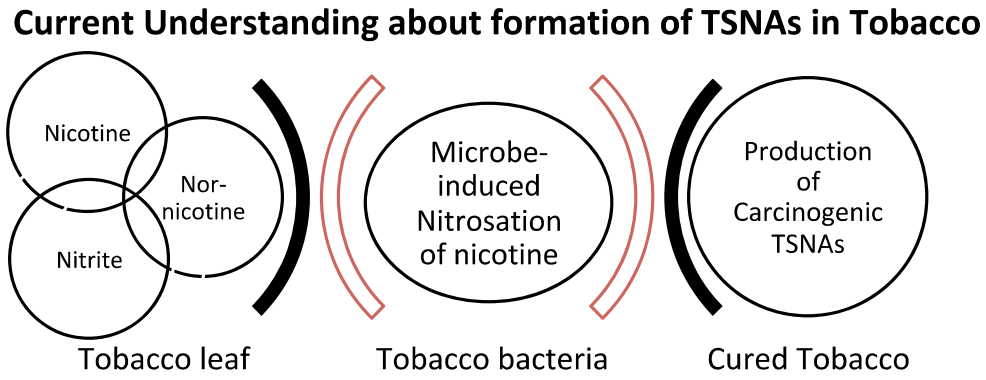

Fig 2: Role of microbes in inducing tobacco carcinogenesity

Increasing evidence suggests that along with physiochemical environment, metabolically active microbial communities associated with tobacco leaves play a critical role in nitrosamine formation. Many of these microbes participate in nitrate/nitrite metabolism during curing, storage, and product aging and convert nitrate to nitrite generating nitrite pool. These microbial activities influence biochemical conversion of tobacco alkaloids into carcinogenic TSNAs. However, despite recognizing the role of tobacco microbial populations in TSNAs generation, comprehensive characterization of active microbial community in Indian SLT products remain limited [16, 17].

In this context, the present study begins with isolation of cultivable microflora from commonly consumed Indian SLT products using conventional microbiological approaches, followed by functional screening and characterization of nitrate/ nitrite reducing microbes present in Indian SLT products for the first time. Further, Antibiotic susceptibility profiling of isolated microbes was done to assess any potential environmental and public health concerns associated. This investigation aims to improve current understanding of the interplay between product characteristics, microbial ecology, and TSNA formation. This study further provide a foundation for future studies integrating advance omics-based tapproches such as metagenomics to elucidate microbial conribution to carcinogenecity in SLT products.

2. Methodology:

Eighteen SLT products of different categories of Pan Masala, Khaini, Zarda, Chewing tobacco leaves, Dohra, Gudhku, Kiwam and toothpowder were procured from local shops in Delhi, Noida and various locations of Uttar Pradesh as represented in Figure 3 and Table 1. Each category of the product has three different variants. The unopened packages were stored at 4°C before analysis. Material of pouches were opened and pooled for the analysis.

2.1. Isolation and cultivation of Microbes:

The conventional microbiological methods were applied to identify and characterize the cultivable bacteria from SLTs as shown in Figure 3. In short, 1 gm of tobacco samples were dispersed in 10 ml Phosphate Buffer (0.1 M, pH 7), kept on shaker for 1 hr at room temperature (28-30°C) and centrifuge at 800 rpm for 10 minutes to remove the debris. For quantitative assessment, serial dilution protocol was followed and after standerdizing appropriate dilution required for each samples, 100 µl from final dilution was spread on agar plates and incubated 37°C (48 hours for

Table 1. Smokeless Tobacco Samples (SLTs) Used in this study (ND* Not Done)

Sr. No.	SLT product	Place of Procurement	Product type and use	No of microbes isolated	Tobacco and additives used
1	SLT1	Prayagraj	Pan Masala chewed separately or with pan	213	Dried tobacco leaves with spices, flavouring additives, and silver flakes
2	SLT2	Prayagraj	- do-	216	- do-
3	SLT3	Delhi/ NCR	- do-	258 (MAX)	- do-
4	SLT 4	Prayagraj	Khaini for dipping	18	Sun dried tobacco leaves to be used with slaked lime
5	SLT 5	Prayagraj	- do-	51	- do-
6	SLT 6	Prayagraj	- do-	38	- do-
7	SLT 7	Delhi/ NCR	Zarda added to pan and chewed	89	Boiled tobacco leaves with spices, flavouring additives, and silver flakes
8	SLT 8	Prayagraj	- do-	15	- do-
9	SLT 9	Delhi/ NCR	- do-	2(MIN)	- do-
10	SLT 10	Prayagraj	Tooth powder rubbed over the teeth and gum	206	Fine powdered tobacco in tooth powder
11	SLT 11	Prayagraj	Gudakhu rubbed over the teeth and gum	76	Coarse, wet tobacco paste with molasses, lime, red soil, and various ingredients
12	SLT 12	Mainpuri	Kiwam Added to pan and chewed	202	Fine paste of tobacco extract, spices, and additives
13	SLT 13	Delhi/ NCR	Tobacco leaves for chewing with Pan masala or with Pan	87	Tobacco leaves to be used with Pan Masala product
14	SLT 14	Prayagraj	- do-	72	- do-
15	SLT 15	Prayagraj	- do-	17	- do-
16	SLT 16	Prayagraj	Local variety of Dohra for chewing	216	Tobacco leaves mashed with various ingredients
17	SLT 17	Pratapgarh	- do-	ND*	- do-
18	SLT 18	Pratapgarh	- do-	ND*	- do-

Fig 3: Different varieties of SLTs used in north India

bacteria on Nutrient agar (NA); 7 days for fungi on Potato Dextrose Agar (PDA)) and the colony-forming units were calculated. Pure colonies were isolated through quadrilateral streaking and stored on agar slants (Figure 4).

Isolated microbes were grown on Nitrate/Nitrite specific media for isolating nitrate/nitrite reducing microbes. Nitrate/Nitrite reducers were assassed spectrophotometrically at 420 nm and 540 nm, respectively for their capacity to reduce Nitrate/Nitrite as described by Buxton et al 2011 [18].

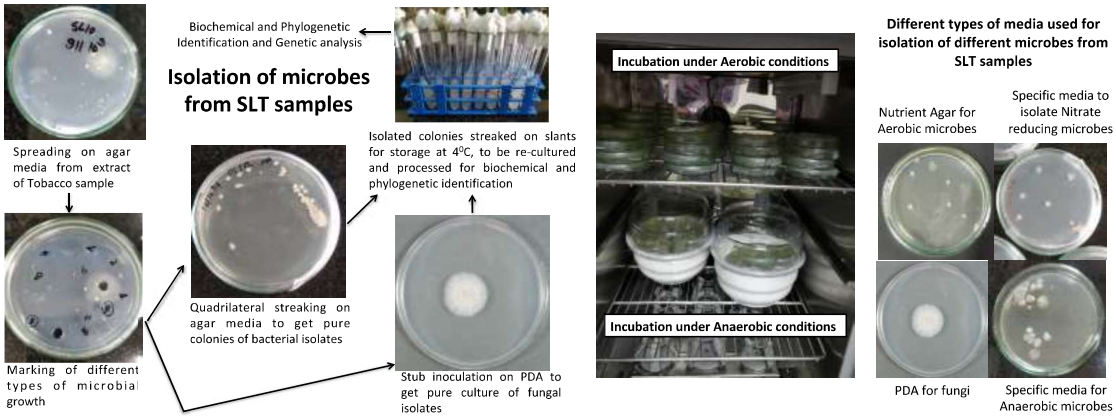

Fig 4: Isolation of cultivable microbes from SLTs

2.2. Biochemical and Metabolic profiling of Isolates:

Gram staining was was done followed by 11 biochemical tests as shown in Figure 5, including Citrate, Lysine, and Ornithine utilization, Phenylalanine deamination, Urease, and Nitrate reduction

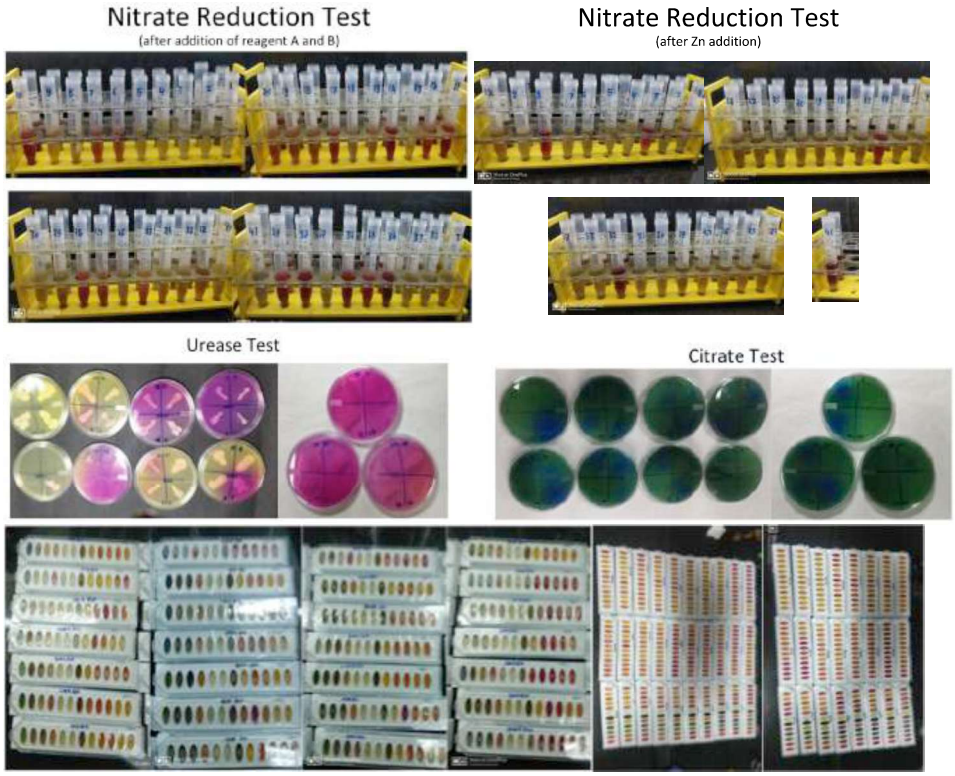

Figure 5: Some of the biochemical tests

using KB002 HiAssorted Biochemical Test Kit from HiMedia. Carbohydrate utilization was tested using KB0009A,B&C colorimetric Himedia kit as per the manufacturer instructions. Carbohydrates studied were - Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose, Mannose, Inulin, Sodium gluconate, Glycerol, Salicin, Dulcitol, Inositol, Sorbitol, Mannitol, Adonitol, Arabitol, Erythritol, alpha-Methyl-D-glucoside, Rhamnose, Cellobiose, Melezitose, alpha-Methyl-D-Mannoside, Xylitol, ONPG, Esculin, D-Arabinose, Malonate and Sorbose.

Antibiotic resistance was determined using the Kirby-Bauer disk diffusion method with 10 different antibiotics (Chloramphenicol, Ampicillin, Gentamicin, Neomycin, Penicillin G, Streoptmycin, Erythromycin, Kanamycin, Polymyxin–B, Nalidixic Acid) and absence of growth area around the disks was recorded.

3. RESULTS

Smokeless tobacco products (SLTs) is a common name given to heterogenous group of products available for use without burning tobacco. The SLTs are commonly taken orally by chewing or sometimes by sniffing through nose as snuss and Indian Naswar. Oral use of SLTs are common among indians. We collected 18 different types of SLTs from north India (Figure 3) and studied them for the presence of microbial components (Table 1).

3.1. Microbial parameters:

Study of 18 SLTs resulted in isolation of 1776 distinct microbial colonies (Table 1) from which 35 Nitrate/Nitrite reducing bacteria (Table 2 and Figure 6) were isolated as pure culture. Cultivable microbial community composition was highly variable among groups studied. Four types of microbial communities i.e. anaerobic, aerobic, nitrate/nitrite reducing bacteria and fungi groups were studied among all the samples.

Table 2: Nitrate/Nitrite reducer Microbes

Group Number	Sample Type	No of Nitrate/Nitrite reducing microbes Isolated
Group 1	Pan Masala Samples	9
Group 2	Khaini/Surati Samples	5
Group 3	Zarda Samples	8
Group 4	Heterogenous Group	3
Group 5	Chewing tobacco leaves	10

As presented in Figure 6, it was observed that all the Pan masala samples (SLT 1-3) and Chewing tobacco leaves (SLT13-15) were richly harbouring all groups of microbes studied whereas Khaini (SLT4-6) only showed anaerobic microbes. None of the Khaini samples showed presence of aerobic, fungal or nitrate/nitrite reducing microbes. Zarda samples (SLT 7-9) also represented poor presence of microbial population. None of Zarda samples showed presence of fungi. None of the samples from Heterogenous group (SLT10-12) having one toothpowder, one Gudaku and one Kiwam sample showed presence of anaerobic microbes. Two samples (SLT 11 and 12) also lacked presence of fungi. Chewing tobacco leaves samples (SLT 13-15) were next best in carrying cultivable microbial microbial load. All samples of chewing tobacco leaves richly represented presence of nitrate/nitrite reducing microbes along with aerobic and anaerobic microbes however, none of them showed any representation of fungal group.

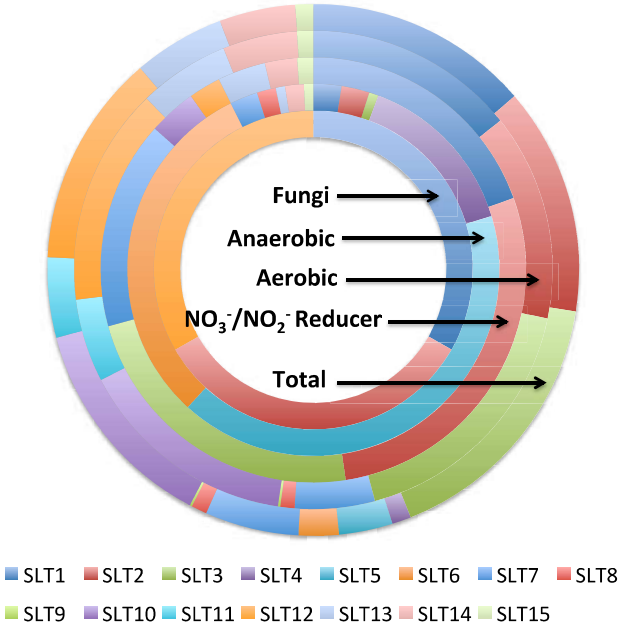

Fig 6: Types of Microbes isolated from SLT samples studied

Out of 1776 isolates; 35 microbes showing nitrate and/or nitrite reducing capabilities were selected out from 18 different SLTs studied (Table 2). All bacterial isolates were found to be good nitrate reducers except isolate number 1 as presented in figure 7A. Out of 35 isolates, 12 isolates were found to be good nitrite reducers as presented in figure 7B .

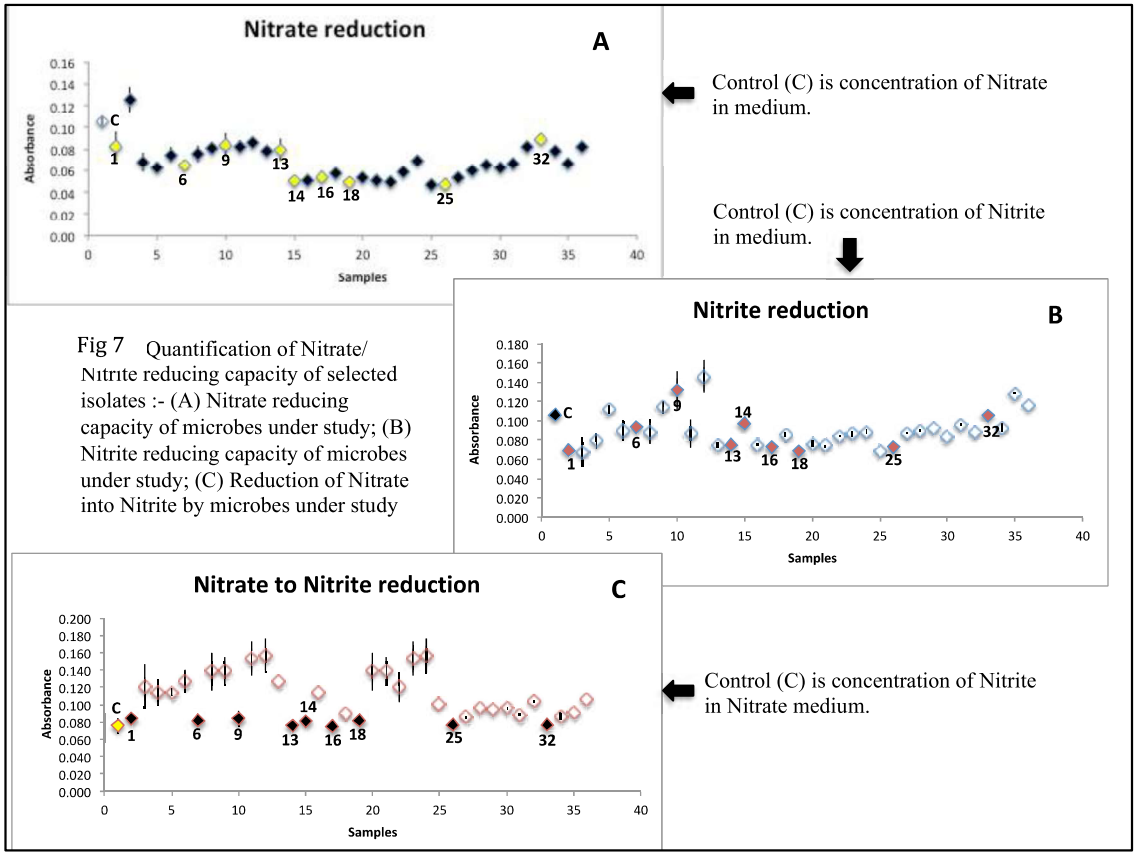

Fig 7 Quantification of Nitrate/ Nitrite reducing capacity of selected isolates :- (A) Nitrate reducing capacity of microbes under study; (B) Nitrite reducing capacity of microbes under study; (C) Reduction of Nitrate into Nitrite by microbes under study

Fig 7: Quantification of Nitrate/Nitrite reducing capacity of microbes

On further testing of these 9 microbes (isolate number 1, 6, 9, 13, 14, 16, 18, 25 and 32 as presented in figure 7C), isolates 9, and 32 were showing lower capabilities for nitrate and nitrite reduction both as apparent from fig 6A and 6B. Isolates 6 and 14 were having high nitrate reducing capabilities but comparatively lower nitrite reducing capacity. Low nitrate but high nitrite reducing isolates 1 and 13 were shortlisted as isolates of second choice. Isolates 6, 9, 14 and 32 were not included in list for further studies. Out of these, microbes having high capability to reduce nitrite and /or having capability to reduce nitrate completely without converting nitrate to nitrite were to be shortlisted, hence isolates 16, 18 and 25 presenting high nitrate and high nitrite reduction can be investigated further for their role in impacting the quality and quantity of carcinogenic TSNAs in SLT products.

3.2. Biochemical parameters:

These various substrate utilization tests are a part of battery of tests to identify microbes from environmental isolates as presented in Table 3. The production of these various enzymes along with carbohydrate utilization pattern is taken as an important parameter for the differentiation and characterization of isolated microbes. These tests as shown in figure 5 and table 3 indicate the production of particular enzymes hence capability of microbes to utilize that particular chemical to produce energy and grow on medium containing these specific chemicals. The production of these enzymes is taken as an important parameter for the differentiation of bacteria based on their metabolic characteristics.

All the 35 strains selected were nitrate reducer as indicate after nitrate reduction test. All were motility, Indole and H2S production negative isolates. All the microbes were phenylalanine deamination tests negative except isolate number 26 and 33 indicating their disability to produce the enzyme phenylalanine deaminase. Most of them were lactose non fermentor except isolates 2, 4, 15, 28 and 30 indicating they are not from coliform family. Most of the microbes were catalase positive except five isolates. Out of 35 isolates studied, 63% were able to produce urease and utilize glucose from the media while 37% were lacking these abilities. Citrate utilization capability was present in 46%, lysine utilization capability was shown by 31% and ornithine utilization capability was reported by 23% of isolates.

3.3. Antibiotic Resistance patterns:

Antibiotic resistance pattern of all the 35 SLT isolates were studied for 10 different types of antibiotics (Figure 6) to ensure that selected isolates are not exceptionally resistance to common use antibiotics. This study ensure that none of the isolates has any potential threat to environment, animals and humans in general.

Most of the isolates showed sensitivity towards the antibiotics studies. All the 35 isolates were susceptible or moderately susceptible to antibiotics chloramphenicol, gentamycin, neomycin and penicillin G. All the isolates were reported sensitive or moderately sensitive to chloramphenicol, gentamycin, neomycin and penicillin as shown in the figure 6 and Table 3. All except one isolate each were susceptible to antibiotics amphicilin and nalidixic acid. Maximum resistance was recorded against erythromycin where 8 isolates reported resistant. Streptomycin and kanamycin resistance was observed in five different isolates each, placing them second most resistant antibiotics for the isolates studied while three different isolates showed resistance to Polymixin B.

Five isolates showed resistance to more than one antibiotics compared to others. Isolate no 1 was reported as maximum resistant isolate, showing resistance to three antibiotics- erythromycin, kanamycin and nalidixic acid. Isolate no 11, 12 and 21 were found to show resistant against two anibiotics, erythromycin and kanamycin and Isolate no 15 was resistant to streptomycin and polymixin B.

Table 3: Biochemical parameters and sugar utilization (+Positive, -Negative, D Doubtful)

Isolate number	Citrate Utilization	Lysine Utilization	Ornithine Utilization	Urease	H2S Production	Glucose Utilization	Adonitol	Lactose	Arabinose	Sorbitol	Motility	Catalase production	Indol test	ONPG	Phenylalanine Deamination
1	+	-	-	+	-	+	-	-	+	+	-	-	-	-	-
2	-	+	-	+	-	+	-	+	+	+	-	+	-	-	-
3	-	-	-	+	-	+	-	-	+	+	-	+	-	-	-
4	-	+	-	+	-	+	-	+	+	+	-	+	-	-	-
5	+	-	+	-	-	+	-	-	-	-	-	+	-	+	-
6	+	-	+	-	-	-	-	-	-	-	-	+	-	+	-
7	+	-	+	+	-	-	-	-	-	-	-	+	-	+	-
8	+	+	-	+	-	+	-	-	+	D	-	-	-	+	-
9	-	+	-	+	-	+	-	-	D	+	-	+	-	+	-
10	+	-	-	+	-	+	-	-	+	+	-	+	-	+	-
11	-	+	-	+	-	D	-	-	+	+	-	+	-	-	-
12	+	+	-	+	-	+	+	-	+	+	-	+	-	+	-
13	+	-	-	-	-	+	-	-	+	-	-	+	-	-	-
14	+	-	-	-	-	+	-	-	+	-	-	+	-	-	-
15	-	+	+	+	-	D	D	D	+	D	-	+	-	-	-
16	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-
17	+	-	-	-	-	+	-	-	+	D	-	+	-	-	-
18	-	-	-	+	-	-	-	-	D	D	-	+	-	-	-
19	+	-	-	-	-	+	-	-	-	-	-	+	-	+	-
20	+	-	+	-	-	D	-	-	+	+	-	+	-	-	-
21	+	+	-	-	-	+	-	-	+	-	-	+	-	-	-
22	-	+	-	+	-	+	-	-	+	+	-	+	-	+	-
23	-	+	-	+	-	D	-	-	D	-	-	-	-	+	-
24	-	-	-	-	-	+	-	-	D	+	-	+	-	+	-
25	-	-	-	-	-	+	+	-	D	+	-	+	-	+	-
26	-	-	-	-	-	+	-	-	+	+	-	+	-	-	+
27	+	-	-	-	-	+	D	-	+	+	-	+	-	+	-
28	+	-	+	-	-	-	-	+	+	+	-	-	-	-	-
29	+	-	-	+	-	+	D	-	+	+	-	+	-	+	-
30	+	-	-	+	-	-	-	+	-	-	-	-	-	+	-
31	-	-	-	+	-	D	-	-	-	+	-	+	-	+	-
32	+	-	+	+	-	-	D	-	-	+	-	-	-	+	-
33	-	-	+	+	-	+	D	-	+	+	-	+	-	+	+
34	-	-	-	+	-	D	-	D	+	-	-	+	-	+	-
35	+	-	-	+	-	+	-	+	+	D	-	-	-	-	-

Isolate number	Xylose	Maltose	Fructose	Dextrose	Galactose	Raffinose	Trehalose	Melibiose	Sucrose	L-Arabinose	Mannose	Inulin	Sodium gluconate	Glycerol	Sorbose
1	-	+	+	+	-	-	+	-	+	+	+	+	-	-	-
2	-	-	+	+	D	D	+	-	+	-	-	-	-	D	-
3	+	+	D	+	-	-	-	-	-	-	-	+	D	+	-
4	+	+	+	+	+	D	+	-	+	+	+	+	+	+	-
5	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	+	D	D	+	-	-	D	-	+	+	+	D	-	D	-
9	-	-	+	-	-	-	-	D	D	D	D	-	-	-	-
10	-	-	-	+	-	-	+	-	+	+	+	+	-	-	-
11	-	+	+	D	+	-	-	-	+	+	+	+	+	+	-
12	-	-	-	-	D	-	-	-	+	+	+	+	-	+	-
13	-	+	+	+	-	-	+	-	+	+	+	-	-	-	-
14	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-
15	D	+	+	+	D	D	-	-	+	+	-	+	D	+	-
16	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-
17	-	-	-	D	-	-	+	-	+	+	+	-	-	-	-
18	-	-	-	-	-	-	-	-	+	D	+	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	+	+	+	-	-	+	-	+	+	+	+	-	-	-
21	+	+	+	-	+	-	+		+	+	+	D	D	-	-
22	+		+	+	+	+	+	D	+	+	+	+	D	-	-
23	D	+	+	-	-	-	+	-	+	D	+	+	-	-	-
24	-	+	+	+	-	-	+	-	+	D	+	+	-	-	-
25	D	D	+	+	D	-	+	D	+	D	-	+	-	+	-
26	+	+	+	+	+	-	+	-	+	+	+	+	+	+	-
27	+	+	+	+	D	D	+	-	+	+	-	+	D	+	-
28	+	+	+	+	+	-	+	-	+	+	+	+	D	-	-
29	+	+	+	-	D	D	+	-	+	+	+	-	-	D	-
30	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
31	-	-	+	+	-	-	+	-	+	-	+	+	-	-	-
32	-	+	+	+	+	-	+	-	+	-	-	+	-	-	-
33	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
34	+	+	+	+	+	-	+	-	+	-	-	+	+	+	-
35	+	+	+	+	+	+	+	D	+	+	+	-	-	-	-

Isolate number	Salicin	Dulcitol	Inositol	Sorbitol	Mannitol	Erythritol	alpha-Methyl-D-glucoside	Rhamnose	Cellobiose	Melezitose	alpha-Methyl-D-Mannoside	Xylitol	Esculin	D-Arabinose	Malonate
1	+	-	+	+	+	-	-	-	+	-	+	-	+	+	-
2	+	-	-	+	+	-	-	-	-	-	+	-	+	+	-
3	+	-	-	+	+	-	-	-	+	-	+	-	+	+	-
4	+	-	-	+	+	-	-	+	+	-	+	-	+	+	-
5	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
8	D	-	-	D	D	-	-	-	-	-	+	-	+	-	-
9	-	-	D	+	-	-	-	-	-	-	+	-	+	-	-
10	-	-	-	+	-	-	-	-	-	-	+	-	+	-	-
11	-	-	-	+	+	-	-	-	+	-	+	-	+	-	-
12	-	+	+	+	+	+	+	+	+	-	D	-	+	-	-
13	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-
14	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-
15	+	-	D	D	+	D	+	-	-	-	+	-	+	D	-
16	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-
17	-	-	-	D	+	-	-	-	+	-	+	+	+	-	-
18	+	-	D	D	+	-	-	-	-	-	-	-	+	-	-
19	-	-	-	-	-	D	D	D	+	-	D	-	+	D	-
20	+	-	-	+	+	-	-	-	+	-	-	-	+	-	-
21	-	-	-	-	+	-	-	-	+	-	+	-	+	+	-
22	+	-	D	+	-	D	D	D	+	-	D	-	+	+	-
23	-	-	-	-	-	-	-	D	+	-	D	-	+	-	-
24	-	-	+	+	+	-	-	-	+	-	D	-	+	-	-
25	D	+	+	+	+	+	+	+	+	-	+	-	+	-	-
26	+	+	-	+	+	-	-	-	+	D	+	D	+	-	-
27	+	-	D	+	+	D	-	-	D	-	D	-	+	+	-
28	+	-	D	+	+	-	-	D	+	+	-	+	+	+	-
29	+	D	+	+	+	D	D	-	+	-	+	-	+	-	-
30	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
31	+	-	-	+	+	-	-	-	+	-	-	-	+	-	-
32	-	+	+	+	+	D	D	-	-	-	-	-	+	-	-
33	+	D	+	+	-	D	D	-	-	-	+	-	+	-	-
34	+	D	-	-	-	-	-	-	+	-	-	-	+	-	-
35	+	D	D	D	D	-	-	-	+	-	-	-	+	-	-

Figure 8: (A) Antibiotic disk details (1=Chloramphenicol, 2=Ampicillin, 3=Gentamicin, 4=Neomycin, 5=Penicillin G, 6=Streoptmycin, 7=Erythromycin, 8=Kanamycin, 9=Polymyxin B, 10=Nalidixic Acid

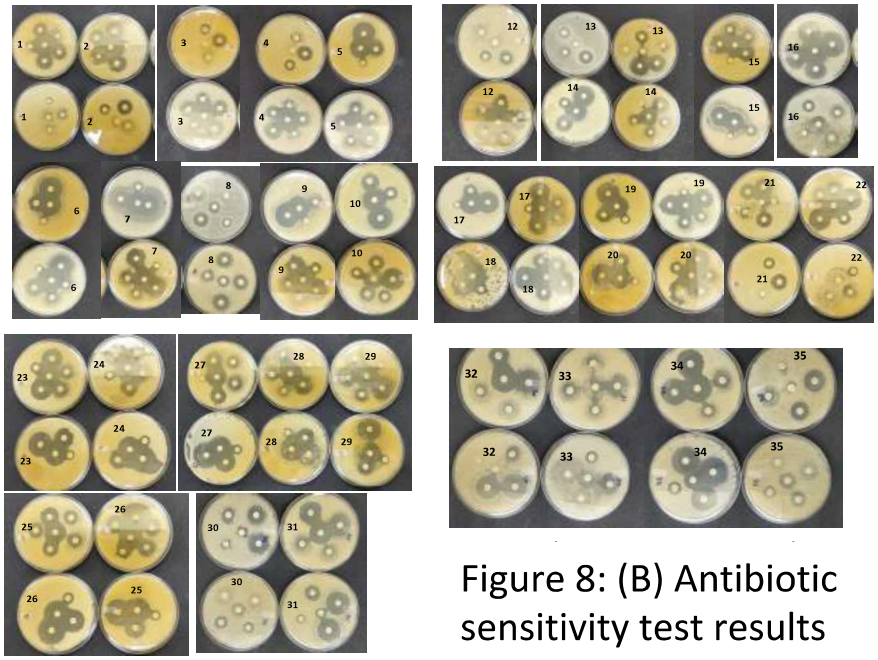

Figure 8: (B) Antibiotic sensitivity test results

Table 4: Antibiotic sensitivity test results (S=Susceptible, M= Moderately susceptible, R= Resistance)

Antibiotics	Symbol	Disc content	Clearence around disk	1	2	3	4	5	6	7	8	9	10	11	12	13
Chloremphenicol (30mcg)	C 30	30 mcg	R>12mm	S	S	S	S	S	S	S	S	S	S	S	S	M
Amphicilin (10mcg)	AMP 10	10 mcg	R>13mm	S	M	S	S	S	S	S	S	S	S	S	M	R
Gentamycin (10mcg)	GEN 10	10 mcg	R>12mm	S	S	S	S	S	S	S	S	S	S	S	S	S
Neomycin (30mcg)	N 30	30 mcg	R>13mm	S	S	S	S	S	S	S	S	S	S	S	S	S
Penicillin-G (10units)	P 10	10 units	R>13mm	S	S	S	S	S	S	S	S	S	S	S	S	M
Streptomycin (10mcg)	S 10	10 mcg	R>11mm	S	S	S	S	S	S	M	S	S	S	S	S	S
Erythromycin (15mcg)	E 15	15 mcg	R>13mm	R	S	R	R	S	S	S	R	S	S	R	R	S
Kanamycin (30mcg)	K 30	30 mcg	R>13mm	R	S	R	S	S	S	S	S	S	S	R	R	S
Nalidixic Acid (30mcg)	NA 30	30 mcg	R>13mm	R	S	S	S	S	S	S	S	S	S	S	S	S
Polymixin-B (300units)	PB 300	300 units	R>13mm	M	S	S	S	M	M	M	S	R	S	S	S	S

Antibiotics	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Chloremphenicol (30mcg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	M
Amphicilin (10mcg)	M	S	S	S	S	S	S	M	S	S	S	S	S	S	M	S	S	S	S	S	S	M
Gentamycin (10mcg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Neomycin (30mcg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Penicillin-G (10units)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Streptomycin (10mcg)	R	R	S	S	S	M	S	S	S	S	S	S	S	M	R	R	S	S	R	S	S	S
Erythromycin (15mcg)	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R
Kanamycin (30mcg)	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Nalidixic Acid (30mcg)	S	S	S	S	S	S	S	S	M	S	S	S	S	S	S	S	S	S	S	S	S	S
Polymixin-B (300units)	S	R	R	M	S	M	M	S	M	M	M	M	M	M	M	M	M	S	S	S	S	S

4. Discussion

Indian SLT varieties are largly manufactured under heterogeneous and largely unregulated conditions in local or cottage industries [19]. The variety of products and absence of standardized manufacturing practices contributes to marked differences in physiochemical, and microbiological composition across Indian SLT products. Variability in formulation (Khaini, Kiwam, Dohra, Pan masala etc), additives (lime, flavoring agents, and areca nut derivatives) and processing techniques results in substantial difference in appearence, moisture content, alkalinity and microbial content [1, 20]. These factors collectively influence nicotine bioavailability, microbial survival, and TSNAs formation [15, 16].

Traditionally we all know tobacco is bad because it contain Nicotine that is harmful for health and it is addictive. Nicotine is a toxic and intoxicating substance that is quickly absorbed through the skin and mucous membranes in its basic unionized form resulting in stimulation thus addiction. However, TSNAs not Nicotine are a likely cause of tobacco-induced cancers and other diseases resulting into morbidity and mortality. Fresh tobacco leaves contain high content of tobacco alkaloids as nicotine and nitrate but very low N-nitrosamine compounds. Diverse microbial communities present in and around tobacco leaves contribute to the formation of these harmful chemical byproducts [4, 15-17]. Thus there is a critical need to investigate micobial composition of tobacco using conventional microbiological approaches to understand the active microbial component and their role in the formation of carcinogenic N-nitrosamine compounds (NNN and NNK etc).

4.1. Microbial properties:

The cultivable microbes represent the part of microbial communities which can be grown and analyzed under laboratory conditions. Conventional microbial methods were used for counting and isolation of cultivable microbes from SLTs [1, 21, 22]. Total 1776 different types of Cultivable Aerobic microbes, Anaerobic microbes, Nitrate/Nitrite reducing microbes and Fungi were counted from 18 SLT samples inpresent study out of which 35 Nitrate/Nitrite-reducers were identified, to understand the microbial community structure of SLTs. Earlier studies reported presence of Nitrate/Nitrite-reducers in SLT products and discussed their role in in the formation of carcinogenic TSNAs [21] however, present study is first of its kind confirming presence of metabolically active Nitrate/Nitrite-reducers in Indian SLT products.

As all the Khaini samples reporting present of anaerobic microbes only, were packed with lime (Calcium Carbonate, $CaCO3$) that may create higher concentration of Carbon di oxide ($CO2$) inside the packages. This high $CO2$ environment may have supported presence of anaerobic microbes and supressed livability of other types of microbes requiring Oxygen ($O2$) for their survival. However, these observations require further detailed investigation and experimental validation because other studies reported fungal presence in Khaini [1, 20].

Zarda samples of also represented poor presence of microbial population. None of Zarda samples showed presence of cultivable fungi though omics related studies reported presence of fungal component in Zarda samples [23]. It may be due to presence of higher chemical load or due to standardized and controlled manufacturing conditions. All the Zarda samples were from branded companies boasting compliance to various international and national food security standards. Standardized sanitary conditions at factory level manufacturing may also contribute to reduced microbial load however, these possibility needs further detailed investigation.

The heterogenous group having one toothpowder sample, one Gudaku sample and one Kiwam sample showed variable microbial spectrum [1,20] suggesting production related processing and additives drive microbial load in Indian SLT products in absence of standerdized and regulated manufacturing framework.

Chewing tobacco leaves samples were next best after Pan Masala samples in carrying cultivable microbial load. All samples of chewing tobacco leaves and Pan masala richly represented presence of nitrate/nitrite reducing microbes along with aerobic and anaerobic microbes [1, 20]. Pan Masala samples showed presence of fungal varities also [23] however, none of the chewing tobacco leaves showed any representation of fungal group. Chewing tobacco leaves samples were the least processed SLT products with minimal additives. As both the products contain substaintial amount of tobacco leaves as base products, that might be the reason for shared microbial profile. Higher microbial load in Pan Masala Samples may be due to contaimination ocurring during post production handling. Addition of multiple additives as spices etc could also introduce microbial contaimination. Limited regulatory enforcements and inadequate supervision during manufacturing may also contribute to increased microbial contamination [19].

Despite the diverse microbial ecology observed across SLTs studied, some loose trends may be seen as all Khaini samples showed only representation of anaerobic microbes and all Pan masala samples showed good representation of all groups of cultivable microbes. Chewing tobacco leaves samples were next best in harbouring three groups of cultivable microbes including nitrate/nitrite reducing microbes, aerobic and anaerobic microbes except anaerobic group. These results showed no homogenous presence of microbes among SLT samples confirming heterogenous nature of SLTs.

4.2. Nitrate/Nitrite reduction:

Cultivable microbial community structure was highly variable among groups studied. Total 35 nitrate/nitrite reducing bacteria were isolated from various SLTs studied as shown in Table 4. To elucidate the possible types of nitrate/nitrite reducing microbes present in different varieties of SLTs, nitrate/nitrite specific media were used. This was first step towards understanding and establishing the impact of microbial population towards generation of TSNAs and other associated chemicals present in SLTs. So far no studies have been done in India to establish the impact of microbial population on Tobacco Specific Nitrosamines and other associated chemicals present in smokeless tobacco products.

Out of those 35 Nitrate/Nitrite-reducing isolates, all were nitrate reducers and only 12 were good nitrite reducers as shown in Figure 7B. Nine isolates were identified good nitrite-reducer as they utilize nitrate completely without creating a nitrite pool. Chewing tobacco leaves and Pan masala samples contributed to 53.4% of the total nitrate/nitrite-reducing microbes isolated (Table 4). After screening their capabilities to reduce nitrate fully beyond nitrite, nine isolates (isolate 1, 6, 9, 13, 14, 16, 18, 25 and 32 as presented in figure 7C) were found reducing nitrate completely hence utilizing it fully for their metabolic requirements. These isolates were identified as promising candidates as they did not accumulates nitrite during nitrate metabolism thus preventing the formation of nitrite pool, that serves as a key precursor for TSNAs generation as illustrated in Figure 1. These isolates appear to utilize nitrate efficiently and hence possibly reducing both the nitrate and nitrite levels and limiting the availability of this intermediate product required for TSNA formation thus, they may contribute in reducing carcinogenic potential of SLT products [16, 21]. However, further detailed studies are required to substantiate this potential preventive role of nitrate/nitrite reducing microbial isolates.

4.3. Biochemical analysis:

Biochemical tests are used for the suggestive identification of microbes based on the differences in the biochemical activities of different bacteria as according to Bergey's Manual [24]. All the 35 microbial isolates were analysed for 12 biochemical tests for preliminary characterisation and results obtained are summerizes in Table 5. Out of 35 microbial isolates, 5 isolates (number 16, 18, 25, 1 and 13) were found to reduce nitratre completely thus have good affinity in reducing nitrite pool.

Preliminary biochemical characterization of these nitrate-reducing, gram negative isolates demonstrated negative H2S production along with negative motility and negative indole reaction results suggesting that isolates are presumptive non-motile Gram-negative belonging to enterobacteriaceae but are not belonging to pathogenic Salmonella or E coli or Proteus / Morganella group. While such biochemical traits alone cannot conclusively establish non-pathogenicity, they provide an initial indication of low virulence potential of the isolates. To further support this preliminary assessment, antibiotic susceptibility profiling was conducted. Extended carbohydrate utilization pattern showed wide carbohydrate metabolism and ability to utilize plant carbohydratesrepresent typically tobacco associated microflora that has adaptability to survive harsh environmental conditions as curing.

All thirty-five isolates were grouped into four major clusters according to their biochemical and carbohydrate utilization profile [24]. Cluster I consisted of metabolically versatile non-motile Enterobacterales resembling Klebsiella/Raoultella species. Cluster II included environmentally adapted fermentors resembling with Enterobacter/Pantoea species. Cluster III comprised isolates exhibiting restricted metabolic activity and are non-fermentative bacteria. Cluster IV represented atypical Enterobacterales showing variable biochemical responses possiblly due to SLT-associated stress conditions. All these isolated bacterial isolates are nitrate-reducing isolates supporting the potential role of tobacco microbiota in nitrate transformation pathways linked to TSNA formation though further detailed studies are required for confirmation.

Table 5: Bacterial grouping based upon Biochemical and Carbohydrate Utilization tests

Cluster	Isolate Numbers	Key Characteristics	Presumptive Identification	Ecological / Functional Interpretation
Cluster I: Klebsiella–Raoultella group	4, 10, 11, 12, 14, 20, 26, 28, 33, 35	Citrate (+), Urease (+), Indole (**–**), Non-motile, PDA (**–**), broad sugar utilization	*Klebsiella pneumoniae* complex / *Raoultella* spp.	Plant-associated Enterobacterales capable of nitrate metabolism, Potential nitrate reducers contributing to nitrite accumulation and TSNA formation
Cluster II: Enterobacter–Pantoea group	**1**, 2, 3, **13**, **16**, 17, 21, 22, 23, 24, 29	Citrate variable, Urease (+/**–**), Indole (**–**), diverse carbohydrate fermentation	*Enterobacter cloacae* complex / *Pantoea agglomerans*	Environmental fermenters common in plant-derived materials
Cluster III: Low-metabolic environmental bacteria	5, 6, 7, **18**, 19, 30, 31	Limited sugar utilization, weak biochemical activity, nitrate (+)	*Acinetobacter* spp. / related environmental Gram-negative bacteria	Background microbiota with reduced fermentative capacity and limited metabolic contribution
Cluster IV: Atypical Enterobacterales	8, 9, 15, **25**, 27, 32, 34	Delayed/variable reactions, urease variable, irregular carbohydrate profile	Unresolved Enterobacterales spp.	Stress-adapted tobacco microbiota requiring molecular confirmation

4.4. Antibiotic resistance:

Resistance pattern of all the 35 SLT isolates were studied for 10 different types of antibiotics (Figure 4) to ensure that selected isolates are not exceptionally resistance to common use antibiotics. Though detailed taxonomic identification and virulence gene analysis is required to completely rule out pathogenic potential of isolates, absence of multidrug-resistant phenotypes among the tested isolates suggests a relatively lower immediate environmental and public health concern associated with these microbial isolates. Trends arising from this study clearly suggests that none of the isolates has any potential threat to environment, animals and humans in general.

5. Conclusion

The present study provides a foundational evidence supporting the concept that indian SLT products harbour heterogenous physiochemical conditions supporting diverse microbial communities. This study present a complete and comprehensive understanding first time about metabolically active nitrate/Nitrite reducers in indian SLT products.

The results of present study showed no homogenous presence of microbes among SLT samples. Cultivable microbial community structure was highly variable among products studied thus confirming the heterogeneous nature of microbial community structure present in the tobacco products available in Indian market.

Further focuseing on the concept that metabolically active microbes may influence nitrate/nitrite pathways hence affecting nitrite availability for TSNAs production, the study confirmed presence of nitrate/nitrite reducing bacteria in SLTs. Isolates showing efficient nitrate utilization abilities without generating nitrite pools were further characterized and and preliminary safety assessment was performed using antibiotic susceptibility testing as an initial risk-screening strategy. None of the isolates were assesed having any potential threat to environmenta and were suggested to be safe for potential public health concerns.

Considering the exploratory nature of this pilot study, initial biochemical characterization combined with antibiotic resistance profiling were used for screening of relevant nitrate-metabolizing strains, initial characterization and preliminary safety evaluation. The study reported presence of metabolically active nitrate/nitrite reducers in SLT products. Functionally relevant nitrate-metabolizing strains were selected for future in-depth molecular and mechanistic investigations. Further detailed studies are required to investigate microbial contributions to TSNAs biosynthesis and role of nitrite-reducer microbies as potential modulators of TSNA production.

6. Future perspective

The outcome of this work led to multidirectional leads for future prospects as presented in figure 9. One major directions of study may be precise identification of microbial communities associated with nitrate metabolism and in silico toxicokinetic and metabolic pathway analyses using Omics based approches. This will help in understanding the role of microbes in nitrate and nitrite reduction and formation of TSNAs during tobacco processing and storage aimed at developing better processing strategies for developing improved smokeless tobacco formulations and thereby lowering the carcinogenic burden of these products.

Figure 9: Projection of Potentials

Another track of study may be studying the biological implications of microbially-mediated TSNA formation through carcinogenicity-related studies using in vitro cell culture systems, appropriate animal models, and clinical or epidemiological assessments. Such integrated future studies will be able to clarify the complete role of tobacco-associated microbiota in TSNA formation and provide concrete evidence about role of microbes in reducing the health risks associated with smokeless tobacco products.

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