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Prevalence of Moderately Halophytic Aerobic Gram Positive Cocci in Bath Soap Bars with Special Reference to Nesterenkonia Species

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I. Introduction

Soap bars are ideally considered to be free of organisms based on their high alkalinity & salt levels and thus considered not as candidate for spoilage, but repeated incidences of off colours/spots reported over the past has lead to increase incidences of rejection. Thus, in this study 20 unbranded soap bars available in local markets of Mumbai suburbs were screened for the presence of moderately halophilic gram positive cocci with special reference to the lesser known *Nesterenkonia* species, an alkalophilic organism. Eight different media known to support alkalophilic and halophilic organisms used to assess the microbiological load, revealed growth of organisms within the range of 10^3 to 10^5cfu/g. 151 isolates were picked up as representative of Operational Taxonomical Unit (OTU) of which 65 isolates were identified to be gram positive cocci on the basis of their cultural, morphological and biochemical characters. Comparison of 16S rRNA gene sequences showed that 4 isolates were phylogenetically closely related to *Nesterenkonia* species. Thus, survival of an extremophile organism like *Nesterenkonia* within the soap bars require strict imposition of microbiological quality standards and evaluation parameters for a produce that was traditionally thought to be free of organisms.

Soap is a salt of fatty acid [(7)] used as surfactant in conjunction with water for washing and cleaning. Solid bars are obtained through a process of saponification of vegetable or animal oils and fats with a strongly alkaline solution that yields alkali salts of fatty acids (crude soap) and glycerol.

Soap bar making though established by the end of the first millennium, has over the years, evolved from an ordinary cleansing product to one that represents multiple consumer benefits, functionality and even luxury. In modern society due to awareness of hygiene, personal wash (PW) bars has now become a necessity of life. Though 98% of urban household use soap in one form or another [(17)], rural households in India that constitute 70% of the consumer population, is now being targeted with more and more soap brands being launched in the discount segment to target the lower socio-economic strata of consumers [(17)].

A variety of soaps differing in both physical and functional attributes, categorized into perfumed, carbolic and medicated soaps are available in the market. Among these some have herbal ingredients e.g. neem, shikakai, while some have fragrances e.g. rose, gajara etc. Soap due to its alkaline nature has a pH ranging between 7 to 9[(14)]. Based on its high halo-alkaline nature, traditionally most of the soaps were thought not to harbor any microorganisms, however as early as 1955 Billing isolated 31 tolerant organisms from face-flannels and sponges which had many characters in common with *Bacterium anitratum*, *Moraxella* and *Alcaligenes* spp [(2)].

Mollie Mcbride (1984) investigated in use bar and liquid soaps from 5 different washrooms and hand washing station for its microbial load and reported that 92 – 96 % of bar samples and 8 % of liquid samples yielded microbial growth. They reported that in use bar soaps contain microbial load within 0 to 3.8 log CFU per sample and 0 to 2.0 log CFU per sample for liquid soaps. In a similar study, Kabara and Brady (1984) investigated in use bar and liquid soaps from 26 public lavatories wherein 100% bars soaps yielded positive organism with 16 different genera of organisms per bar.

Microbial contamination of in-use bar soaps from dental clinics has been studied by Hegde PP, Andrade AT & Bhat K (2006). They reported that in use soap bars were depots of micro organisms as 100% of samples yielded positive result for the presence of mixed micro flora of gram positive, gram negative, aerobes, anaerobes, and fungi and handwashing with such soap may lead to spread of infection.

Even liquid soap gets contaminated with bacteria and poses a recognized health risk in health care settings. In particular, bulk-soap-refillable dispensers are prone to bacterial contamination, and several outbreaks linked to the use of contaminated soap in health care settings have been reported.[1,3,4,12]

The halo-alkaline soap environments would thus support the prevalence of moderately halophilic microorganisms; prominent being the heterogeneous group of *Micrococcus*, *Nesterenkonia* and *Staphylococcus* genera. This takes on added significance as staphylococci & micrococci are known skin flora capable of surviving in the hypersaline niche within the human skin folds and screening of market soap sample for the presence of these organisms can lead to a better understanding about its prevalence in habitats that are thought to be sterile.

Thus the purpose of this study was to screen the various unbranded personal soap bars available in local markets for the presence of moderately halophilic gram positive cocci and detect among them, the less known *Nesterenkonia* species with an objective to impose micro Quality standards and evaluation parameters in a produce that is traditionally thought to be sterile.

II. Materials & Methods

Bar soap samples: Twenty personal wash soap bars as listed in Table 1 were obtained from the local market. Though unbranded, these soaps had a clean appearance & were well packed.

Nutrient Media: Eight media [18] as listed in Table 2 were selected for total viable counts of alkalophiles and halophiles. Soap noodle agar, an in-house media containing pancreatic digest of casein- 15.0 g/L, enzymatic digest of soyabean meal- 5.0 g/L, sodium chloride- 5.0g/L, agar 15.0 g/L, pH- 7.3 ±0.2, after sterilization of the medium, 10 g/L of commercially available dry heat sterilized soap noodles is added, was also used.

Sample Preparation and Total Viable Count (TVC)

Aseptically, each bar soap was cut in two pieces and one piece was grated using a sterile grater. One gram of soap gratings was then added to 10 ml of neutralizing solution (Phosphate buffer, pH 6.5 containing 0.5% Lecithin soya) to give 1:10 dilution. This was further diluted 1:100 using phosphate buffer diluent (pH 7.0) containing 1% Tween 80. 0.1 ml of each of these dilutions was then used to perform total viable count by spread plate technique on plates of each nutrient media. The plates were incubated at 30^0C for appropriate incubation period as shown in Table 2, with the longest period of 10 days used for soap noodle agar. Colony counts of Gram-positive cocci based on the morphological and gram character was recorded and representative colonies marked as Operational Taxonomical Unit (OTU) were used to identify the strain biochemical. 151 isolates were picked up as representative of Operational Taxonomical Unit (OTU) of which 65 isolates were identified to be gram positive cocci on the basis of their cultural, morphological and biochemical characters.

Identification of Gram positive cocci:

Colonies of Gram positive cocci were differentiated into Micrococci and Staphylococci genera based on aerobic fermentation of glucose in presence of 0.4 µg/ml erythromycin, anaerobic fermentation of glucose, catalase and modified oxidase test (Table 3).

The biochemically identified micrococcus isolates were screened using genus specific PCR primers for identification of *Nesterenkonia* spp based on 16s rRNA gene sequence analysis. PCR with specific primers, Nes 1 (5'- CGC ATA GGG TGC TGG TGG AAA G-3') and Nes 2 (5'-GAG GTC GGG TTG CAG ACT TCG-3')[16] (Sigma Aldrich) was performed in a final volume of 50 µl (Fermentas). Amplification was carried out in a GeneAmp PCR 9700 thermocycler, using the hot- start PCR profile: 4 min at 94^0C for denaturation, and then 30 cycles of 30 s at 56^0C and 2 min at 72^0C, followed by 10 min at 72^0C. Negative controls without template DNA were included for each PCR experiment. Along with the selected isolates, PCR was also carried out using *Nesterenkonia halobia* MTCC 701 and *E. coli* ATCC 25922 as control. Amplification products thus obtained were analyzed by electrophoresis in 1% (w/v) agarose gel and stained with ethidium bromide. Sequencing of the 16S rRNA gene was performed. Phylogenetic affiliation to sequences available in GenBank was determined using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [19].

The phylogenetic analysis of the positive isolates along with 13 standard strains of *Nesterenkonia* was performed using the MEGA (version 5.1) software packages after multiple alignments of data. Clustering was performed by using neighbour-joining method [20]. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1000 resamplings [21].

III. Results and Discussion

Eight different media based on the use of alkaline pH of 10 or addition of salts like NaCl & $MgCl_2$ that allowed the stressed halotolerant & alkalotolerant strains to grow were selected to assess the microbiological load present in soap bars. In spite of such a growth limiting condition, the high viable count on each bar soap was obtained though it varied with the type of medium used. Two distinct category of media could be classified, the first class containing TSYEM & MNM that allowed the recovery of high load of organism ranging within 10^5 to 10^6 cfu per gram from 14 out of 20 of the soap samples analysed & the second containing ELSM, MA, CBA, PYGV media that allowed recovery of low number within 10^1 to 10^4 cfu per gram (Table 4).

Out of 20 soap bars analysed 12.5% of the samples (Swiz No. 1 and Tiems) did not yield count in ELSM while 25% of the samples failed to grow in 2 media out of 8 media used, with CBA featuring prominently in this group. Thus CBA with $MgCl_2$ was the most ineffective media to analyse soap bar sample, as it detected microbial flora in only 6 out of 20 samples tested in contrast to TSYEM & MNM that allowed detection in 18-17 out of 20 samples tested (Table 4). Thus for the assessment of microbiological in terms of heterotrophic loads within bar soaps, a choice of TSYEM, MNM and SNA media can be recommended.

Prevalence of Gram positive cocci in bar soaps:

All the media used in the soap analysis study supported the growth of Gram positive cocci from the soapbars, although the number obtained on each medium differed from 10^3 to 10^5 CFU per gram. 151 isolates were picked up as representative of Operational Taxonomical Unit (OTU) of which 65 isolates could be identified to gram positive cocci / coccoid forms belonging to the Micrococci and Staphylococci genera on the basis of cultural, morphological and biochemical characters (Table 3). Out of which 36 isolates were found to be catalase positive, besides being unable to ferment glucose anaerobically in presence of erythromycin and thus were short listed as belonging to Micrococcus spp.

Phylogenetic analysis:

The 16S rRNA gene sequence of 4 isolates (isolate no. 5, 35, 41 and 56) out of 36 isolates determined them to be closely related to *Nesterenkonia* spp. A phyogenetic tree based on 16S rRNA gene sequences was constructed using the neighbor-joining method (Fig.2) showing Nesterenkonia isolates compared to reference strains. Published sequences from gene bank were used as reference strains (Figure 2) All 4 isolates clustered together indicating that they belong to similar species group *Nesterenkonia halobia* DSM 20451. Out of the 4 isolates 2 isolates showed 100% similarity indicating that similar species are present in different samples.

The presence of large number of Micrococci and Staphylococci in bar soaps raises the question for their very use as personal wash bar soaps. It was interesting to note that soap bar labeled "Elvis" did not show presence of any cocci and for the bar soaps labeled – Cute, Cute Shikakai and Sulphur presence of least number of gram positive cocci was observed, while the maximum number of isolates were found in bar soap labeled as Lamour white, Tiems & an unbranded type. Incidentally no specific isolation media could be attributed solely for the detection of *Nesterenkonia* spp. Thus for the protection of consumers, it is suggested that microbiological quality control with microbial limits using a specific medium such as- TSYEM - pH 10.0 should be introduced for bar soaps specially when Nesterenkonia species, an extremophile could be detected in an environment normally thought to be sterile.

IV. Conclusion

Microbial analysis of Personal wash bars revealed that the soaps itself can harbour microorganisms that leads to incidences of off colours /spots in the soap bars. The present study shows that, *Nesterenkonia* being alkalophile and moderately halophilic is one of the contaminant of Personal wash bars. Thus soaps ideally considered to be free of organism can harbour microbes that can cause its spoilage & increase incidence of rejection. Detection of *Nesterenkonia* in such PW soap takes on added significance as these organisms could be included as one of the indicator organism for the challenge test to be used in screening soap & detergents products.

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Annexure

Table 1: Unbranded Soap Samples collected from local Mumbai market

Soap No.	Sample name	Soap No.	Sample name
1.	Zed	**2.**	Sulphur soap
3.	City beauty soap	**4.**	Sviz no 1
5.	Lamour white	**6.**	Sapna (Ahinsak)
7.	Aimee rose	**8.**	Harmony fruity soap
9.	Tiems soap	**10.**	Elvis
11.	Gold fish soap	**12.**	Jain soap
13.	Cute soap	**14.**	Cute shikakai
15.	Gajara (Ahinsak)	**16.**	Just beauty soap
17.	Healthy soap	**18.**	Unbranded
19.	Limda soap	**20.**	Lamour pink

Table 2: Media Used for analyzing PW Soap Samples

Sr. No	Media	Abbreviation used	pH	Incubation (days)	Specific Ingredient
	Trypticase Soy Yeast Extract Medium	TSYEM	10	4	-
	Modified Nesterenkonia Medium	MNM	7.4	4	10% $MgCl_2.6H_2O$
	Soap Noodle Agar	SNA	7.3	10	1% Soap Noodle
	Ekho Lake Strains Medium	ELSM	7.5	4	Mineral salt solution + Metal solution + Vitamin solution
	Marine Agar	MA	7.6	4	-
	Corynebacterium agar with NaCl	CBA (NaCl)	7.4	4	6% NaCl
	PYGV Agar	PYGV	7.5	4	Mineral salt solution
	Corynebacterium Agar with $MgCl_2$	CBA ($MgCl_2$)	7.4	4	10% $MgCl_2$

Table 3: Differentiation of *Micrococci* and *Staphylococci*

Biochemical Test	*Micrococcus*	*Staphylococcus*
Acid production from glucose under anaerobic condition	-	+
Lysostaphin	R	S
Production of acid from glucose aerobically in the presence of 0.4µg/ml erythromycin	-	+
Furazolidone (100µg furazolidone disk)	R	S

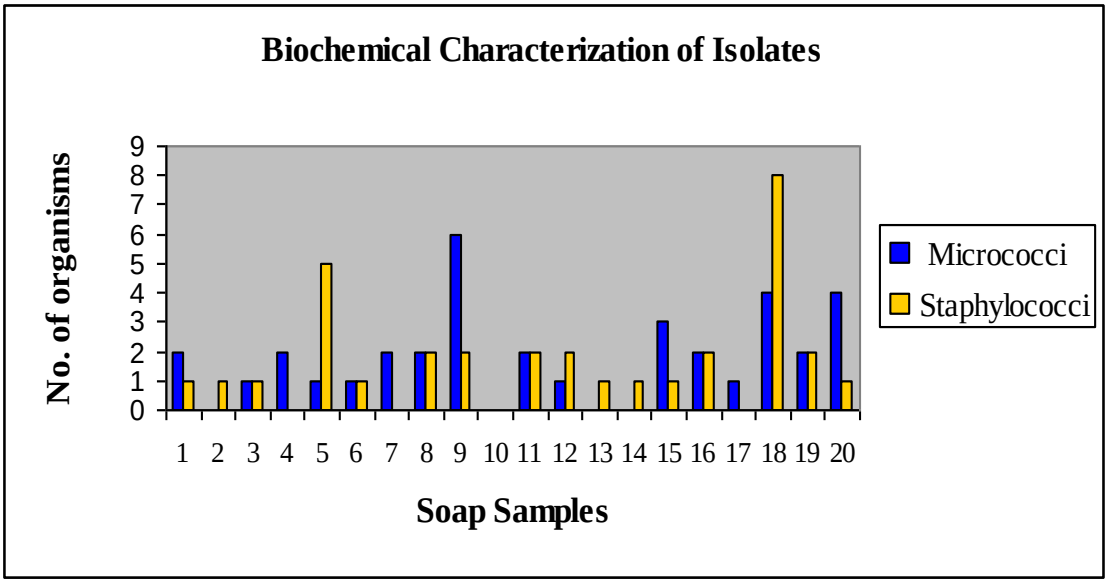
Modified Oxidase test	+	-
Bacitracin (0.04 U Taxo A disk)	S	R

+: positive; - : negative; S: susceptible; R: resistant

Table 4:-Total Bacterial count (CFU per gram) obtained in 20 soaps analyzed in 8 different media

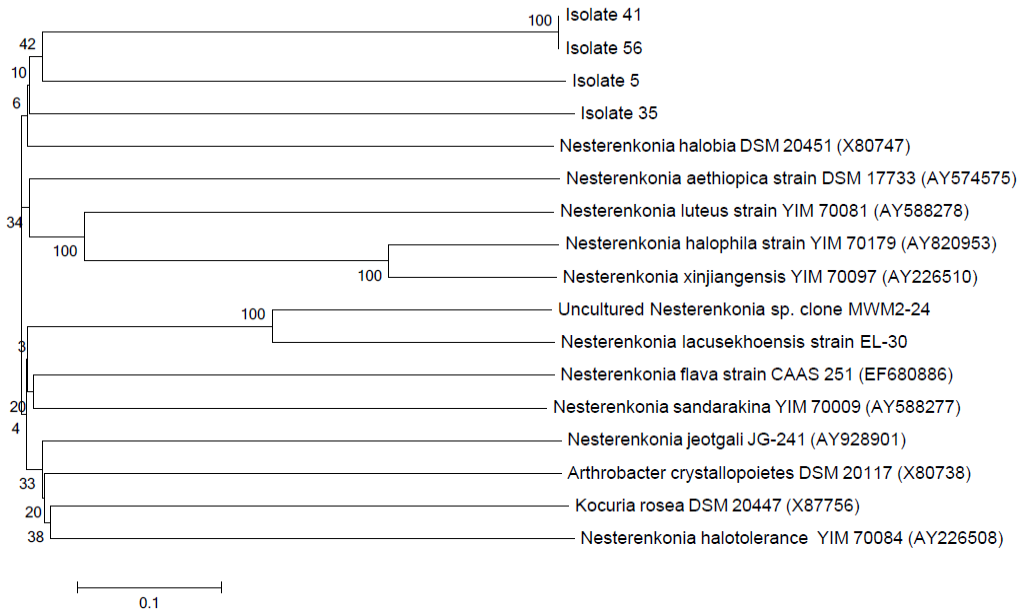
S.No	Media	TSYEM	MNM	SNA	ELSM	MA	CBA with NaCl	PYGV	CBA with Mgcl2
	Soap sample	TVC	TVC	TVC	TVC	TVC	TVC	TVC	TVC
1.	Zed	6.8E+05	1.0E+06	2.0E+03	1.0E+00	1.0E+02	1.0E+02	1.0E+00	1.0E+00
2.	Sulphur	3.0E+06	5.3E+05	3.8E+05	4.0E+02	1.0E+00	5.0E+03	1.0E+03	1.0E+00
3.	City Beauty	3.0E+06	3.0E+06	1.9E+05	1.0E+00	2.1E+03	1.0E+00	4.0E+02	1.0E+00
4.	Sviz no. 1	1.0E+04	2.2E+03	1.8E+05	1.0E+00	3.0E+05	9.0E+03	6.0E+03	1.0E+00
5.	Lamour White	3.0E+06	3.0E+06	1.0E+00	1.0E+00	8.0E+02	1.0E+00	1.0E+00	1.0E+00
6.	Sapna Soap	3.0E+06	3.0E+06	1.0E+00	5.0E+02	3.0E+04	9.0E+03	5.0E+02	1.0E+00
7.	Aimee Rose	3.0E+06	3.0E+06	1.0E+00	1.4E+04	3.0E+03	5.0E+03	1.0E+02	1.0E+00
8.	Harmony	5.1E+05	2.4E+05	3.6E+04	3.0E+05	3.0E+02	3.0E+02	1.0E+00	1.0E+00
9.	Tiems	3.0E+06	2.4E+05	3.0E+05	1.0E+00	1.1E+04	1.0E+03	3.0E+02	1.4E+04
10.	Elvis	3.0E+06	3.0E+06	2.0E+04	7.0E+03	3.0E+04	1.0E+00	1.0E+00	2.0E+02
11.	Goldfish	3.0E+06	3.0E+06	1.0E+00	4.0E+02	1.8E+04	1.0E+02	3.0E+02	1.0E+00
12.	Jain	3.0E+06	3.0E+06	1.0E+00	2.0E+02	2.1E+04	1.0E+00	1.0E+00	1.0E+00
13.	Cute	3.0E+03	1.7E+04	8.0E+03	1.0E+03	6.0E+02	5.0E+02	3.0E+02	5.0E+02
14.	Cute Shikakai	1.0E+03	1.0E+03	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00
15.	Gajara	1.8E+05	1.0E+00	1.0E+04	1.0E+00	7.0E+03	5.0E+03	4.0E+04	1.0E+03
16	Just Beauty	1.0E+00	1.0E+00	2.0E+03	1.0E+00	1.2E+03	1.0E+00	1.4E+03	8.0E+04
17.	Healthy	1.0E+00	1.0E+00	8.0E+05	1.0E+00	3.0E+02	3.1E+03	1.0E+00	1.0E+00
18.	Unbranded	3.0E+05	3.2E+05	1.0E+04	1.0E+00	3.0E+02	3.0E+05	1.0E+00	5.2E+03
19.	Limda	1.1E+06	3.0E+06	1.0E+00	3.0E+02	1.0E+00	1.0E+00	8.0E+03	1.0E+00
20.	Lamour Pink	3.0E+06	3.0E+06	5.2E+04	3.0E+04	6.2E+03	7.0E+03	1.0E+00	1.0E+00

Figure 1: Biochemical Characterization of Isolates for presumptive test of *Micrococcus & Staphylococcus spp.* from Soap Samples



Key: Soap samples: Table 1.

Figure 2: Neighbour-joining tree, based on 16S rRNA gene sequence data, showing the phlogenetic position of Isolates 5, 35, 41, 56 and representative strains. Bootstraps values (1000 replications) are shown as percentages at each node.



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