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Isolation and characterization of oral microflora from Triclosan-free and Triclosan–containing toothpaste users

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Abstract
The mouth harbors a diverse abundant and complex microbial community. This highly diverse microflora inhabits various surfaces of normal mouth. Bacteria accumulate on both the hard and soft oral tissues in Biofilms. Bacteria occupy the ecological niche provided by both tooth surface and gingival epithelium. Oral bacteria include *Streptococci, Staphylococci, Pediococci* and *Enterococci* etc. Triclosan is organic compound which is antibacterial and antifungal agent present in some toothpaste which change the microflora. Saliva was sampled from Triclosan free and Triclosan containing toothpaste users. Different Medias used for isolation of microbes from samples and for characterization various physical and biochemical tests performed. From collected samples various diplococci, rod shaped and cocci shaped bacteria colonies isolated. Further characterization shows that these bacterial colonies belong *to Pediococci, Leuconostoc, Enterococci* and *Streptococci.* But its number is different in Triclosan using toothpaste user's samples and Triclosan free toothpaste user's samples. Percentage of *Streptococci* and *Enterococci* bacteria is more in Triclosan containing tooth paste user's samples and number of *Pediococci* and *Leuconostoc* are more in Triclosan free tooth paste user's samples.

Keywords: Oral microflora, Triclosan, Gram staining, Cell morphology and Chemical test.

1. Introduction
The oral cavity provides a unique environment that supports a wide range of bacterial species. The highly diverse flora grows in the different surfaces found in the mouth. These bacteria found in the mouth are commensals, resident bacteria. They have a symbiotic relationship with the host. These bacteria can be opportunistic. The oral cavity is constantly exposed to changes in the environment. Opportunistic bacteria can take advantage of this and cause disease if the conditions are favourable. Their opportunistic behaviour means that the bacterial growth must but kept under control. Oral flora developed at birth. The oral cavity is sterile during intrauterine life and at birth. Within 8 hours after birth, the mouth acquires organisms. The eruption of deciduous teeth changes the environment in the oral cavity and allows the growth of members of the genus *streptococci.* With increase in the number of teeth and changes in the diet, the oral flora become more complex and more anaerobes become established. Permanent teeth are usually accompanied by dental plaque and chronic periodontal diseases that influence the number and types of bacteria present. Aerobic organisms include *Streptococci, Neisseria* and *Diphtheroids*. Anaerobic bacteria includes *Lactobacilli, Bacteroides, Actinomyces and Veillonella. Candida* is present in 20 – 80% of adults. At old age, with loss of teeth, the number of microorganisms decreases specially: *Lactobacill*i, *Streptococcus mutans* and *Candida.* But with complete dentures usually *Candida* increase again and so do *Streptococcus mutans*.

Streptococci
Streptococci is a genus of spherical Gram-positive bacteria belonging to the phylum Firmicutes and the lactic acid bacteria group. Most *streptococci* are oxidase and catalase negative, and many are facultative anaerobes. *Streptococci* are nonmotile, Gram-positive, non-spore forming bacteria that live in pairs or chains of varying length. They are characteristically round or ovoid in shape. Most Streptococci are facultative anaerobes, although some are obligate anaerobes. *S. mutans* is the primary bacterium involved in plaque formation and initiation of dental caries. *S. pneumoniae* is present in the upper respiratory

tract of about half the population. If it invades the lower respiratory tract it can cause pneumonia. *S. pneumoniae* causes 95 percent of all bacterial pneumonia. *Streptococcus pyogenes* refers to the Group A, Beta-hemolytic streptococci. Streptococci cause tonsillitis (strep throat), pneumonia, endocarditis. *Neisseria* and other Gram-negative cocci are frequent inhabitants of the upper respiratory tract, mainly the pharynx. *Neisseria meningitidis*, an important cause of bacterial meningitis, can colonize as well, until the host can develop active immunity against the pathogen. Lactobacilli in the oral cavity probably contribute to acid formation that leads to dental caries. (Marsh PD *et al.*, 2006) [11].

Enterococci

Enterococci is a genus of lactic acid bacteria of the phylum Firmicutes. *Enterococci* are Gram-positive cocci that often occur in pairs (diplococci) or short chains, and are difficult to distinguish from streptococci on physical characteristics alone (Gilmore MS, *et al.*, ed. 2002) [8]. Enterococci are facultative anaerobic organisms, *i.e.*, they are capable of cellular respiration in both oxygen-rich and oxygen-poor environments (Fischetti VA *et al.*, 2000). Though they are not capable of forming spores, *enterococci* are tolerant of a wide range of environmental conditions: extreme temperature (10-45°C), pH (4.5-10.0) and high sodium chloride concentrations (Fisher K *et al.*, 2009) [6].

Pediococci

Pediococcus is genus of gram-positive lactic acid bacteria of the phylum Firmicutes. Enterococci usually occur in pair or tetrads. They are purely homofermentative. Various species of pediococci are P. acidilactici, P. cellicola and P. damnosus etc. Pediococcus dextrinicus has recently been reassigned to genus Lactobacillus. (Haakensen *et al.*, 2009) [9].

Leuconostoc

Leuconostoc is a genus of gram-positive bacteria, placed within the family of Leuconostocaceae. They are generally ovoid cocci often forming chains. Leuconostoc species are catalase negative. All species within this genus are heterofermentative. They are generally slime forming. (Bjorkroth, *et al.*, 2006) [2].

2. Material and Methods

Sample was collected from saliva of different person. Then 1ml. of samples were placed in test tubes containing 10 ml. of sterile Thioglycolate broth. Samples were mixed properly and also dilutions of samples prepared. Then test tubes were incubated for 3-4 hrs. After incubation portion of dilutions were spread on petri plates containing different media:

- Slantez and Barley medium
- Pfizer Selective Enterococcus
- MRS (Lactobacillus/ selective)
- Acetate (Leuconostoc).

All plates were incubated for 48 hrs at 37 ˚C in CO2 Incubator. After incubation for 48 hrs. Number of viable colonies were counted using total viable plate count method.

C.F.U/mL original sample = Number of colonies /plate x (1/mL aliquot plated) x D.f.

(i) Physical characterization-

Gram staining

The diluted suspensions of the bacteria were smeared on clean slides, air dried, heat fixed by passing over a flame for 2 to 3 times. The slides, were flooded with crystal violet solution for one minute, washed with water and flooded with Gram's iodine for one minute. The slide were washed with water and decolorized with 95% ethyl alcohol dropped from a dropping bottle until no violet colour was visible from drain off solution. The slides were washed with water and counter stained with safranin stain for about 30 second and washed with water. The slides were air dried and examined under a microscope using 100x objectives using a daylight filter. Cells were then identified by the colour observed purple for Gram positive and pink or red for Gram negative cells.

Colony morphology

Shape, size, colour, elevation and margin of colony and appearance are observed in plate culture on different media and noted down.

Cell morphology

The gram stained cells were viewed under light microscope under 100x oil immersion to determine the morphological characteristics of the cells.

Then isolated colonies were then streaked after incubation onto media plates to obtain pure cultures.

(ii) Biochemical tests

Catalase test

The presence of catalase enzyme in the test isolate is detected using hydrogen peroxide. If the bacteria possess catalase (i.e. are catalase-positive), when a small amount of bacterial isolate is added to hydrogen peroxide, bubbles of oxygen are observed. The catalase test is done by placing a drop of hydrogen peroxide on a microscope slide. Pick the colony, and then smears a sample into the hydrogen peroxide drop (Chelikani P *et al.*, 2004) [3].

a) If the mixture produces bubbles or froth, the organism is said to be 'catalase-positive'. Staphylococci and Micrococci (Johnson M., 2009) [10] are catalase-positive.
b) If not, the organism is 'catalase-negative'. *Streptococcus* (Fox A., 2009) [7] and *Enterococcus* spp. are catalase-negative.

Gas productive test

Prepared MRS broth and added 10ml. of broth in sterile test tubes. Placed Durham tube in each of prepared broth in inverted position. This will reveal the presence of any gas-producing bacteria in the samples. Occasionally, air may become trapped in one or more of the Durham tubes. These air bubbles should be removed before the cultures are incubated by inverting the tube gently. Autoclaved the test tubes and incubated for 12-14 hrs. at 37 ˚C. Then add different inoculum in each test tube under sterilized conditions. Then incubate these tubes for 24 hrs. in CO2 incubator. Observed turbidity in test tube and gas bubble formation in Durham tube. Noted the results.

Vancomycin test

Prepared MRS plates. Spreaded the inoculums on plates under sterile conditions. By using Disc diffusion method antibiotic disk of Vancomycin placed on the surface of the medium in plates. Using sterile forceps or a loop, gently press the disks onto the surface of the media, taking care not to press them into media. Invert the plates and incubate for 24 hours at 37 °C in CO2 incubator. Using a scale measure

the diameter of the zone of inhibition (if present) for antibiotic used.

Bile esculin test

Prepared MRS broth in test tubes and autoclaved these tubes. Then added bile esculin disk and inoculums in each test tube. Incubated tubes for 24 hrs. at 37 ˚C in CO2 incubator. A positive result is indicated by a dark brown or black color that diffuses into half or more of the medium. Blackening of less than half of the medium after 48 hours is a negative result.

Temperature stress

Microbes have ability to grow at different temperature. Some microbes grow at high temperature and some microbes grow at low temperature. Checked the growth of microbes at 45 ˚C and 10 ˚C.

Salt stress

NaCl test performed to check salt stress. An inoculum from a pure culture is transferred aseptically to a sterile tube of 6.5% NaCl broth. The inoculated tube is incubated at 35-37 ˚C for 24 hours. A positive test is indicated by the presence of turbidity. Observed test result. The test is positive if there is marked turbidity in the tube. In negative tests, there is no growth.

Preservation in 30% glycerol

Put 700 µl. bacterial culture in a sterile eppendorf tube. Added 300µl of sterile 80% glycerol solution. Freeze on dry ice or directly into –70 ºC. Store at –70 ºC. Cells are best for about 4-6 months, but will probably work ok for a whole year.

3. Results and Discussion

Out of 53 bacterial colonies isolated from both triclosan and triclosan free groups, 4 are diplococci, 1 is rod shape and 48 are cocci. On carrying out the further characterisation of cocci, we found that 14 of them belonged to the genera *Pediococci*, 4 belongs to genera *Leuconostoc/ Weissella*, 7 belongs to genera *Enterococci* and 25 belongs to genera *Streptococci*. Thus, we conclude that the culturable oral flora is dominated by the genera *Enterococci, Pediococci, Leuconostoc/ Weissella and Streptococci.* 5 *Streptococci* isolated from triclosan free group and 20 in triclosan containing group. 3 *Leuconostoc/ Weissella* isolated from triclosan free group and 1 from triclosan containing group. 1 *Enterococci* isolated from triclosan free group and 6 from triclosan containing group. 9 *Pediococci* isolated from triclosan free group and5 from triclosan containing group. Out of 18 triclosan free group, 5.55% *Enterococci*, 27.77% *Streptococci,* 16.60% *Leuconostoc/Weissella* and 50% *Pediococcu*s were obtained. Out of 35 triclosan containing group, 62.50% *Streptococci*, 15.62% *Pediococcus*, 18.75% *Enterococci* and 3.12% *Leuconostoc/ Weissella* were obtained.

Various Tests That Are Uesd for Characterization of Catalase Negative Gram Positive Cocci Are As Follow:

Phenotypic characteristics of facultatively anaerobic, catalase-negative, gram positive cocci.

Genus	Gram[a] stain	Phenotypic characteristic[b]								
		VAN	GAS	BE	PYR	LAP	NaCl	10 ˚C	45 ˚C	HEM
Enterococcus group[c]	Ch	S/R	-	+	+	+	+	+	+[d]	α/γ
Leuconostoc/Weissella[e]	Ch	R	+	V+	-	-	V+	V-	V-	α/γ
Streptococcus	Ch	S	-	-[f]	+	+	V-	-	V-	α/β/γ
Nutritional var. Strep[h]	Ch	S	-	-	+	+	-	-	V-	α/γ
Unusual Strep/Genera[i]	Ch	S	-	V+	V+	V+	V+	V-	V-	α/γ
Pediococcus	Cl/t	R	-	+	+	+	V-	-	V+	A
Tetragenococcus	Cl/t	S	-	+	+	+	+	-	+	A
Aerococcus sps.[j]	Cl/t	S	-	+	V-	V-	+	-	V-	A
Helococcus	Cl/t	S	-	V+	-	-	+	-	-	Γ
Gemella	Cl/t/ch	S	-	-	+	+	-	-	-	Γ
Salt tolerant Gemella-like[k]	Cl/t/ch	S	-	-	+	+	+	-	-	Γ

a. Cell arrangement in gram strain: ch, chains; cl, clusters; t, tetrads.
b. VAN, vancomycin susceptibility screening test; GAS, gas production in MRS broth; BE, hydrolysis of esculin in the presence of bile; PYR, production of pyrrolidonyl arylamidase; LAP, production of leucine aminopeptidase; NaCl, growth in broth containing 6.5% NaCl; 10 ˚C and 45 ˚C, growth at 10 ˚C and 45 ˚C; HEM, hemolytic activity on Trypticase soy 5% sheep blood agar. +, 85% or more of the strains are positive:-, 15% or less of the strains are positive; V+, variable positive (50 to 84% of the strains are positive); V-, variable negative (16 to 49% of strains are positive).
c. Enterococcus group includes all Enterococcus species, Vagococcus species and some Lactococcus species.
d. Some strains of Lactococci and Vagococcus grow very poorly at 45 ˚C.
e. Leuconostoc and *Weissella* are often coccobacillary, sometimes appearing rod like in chains.
f. All strains of S. bovis and approximately 10% of viridians streptococci are bile-esculin positive.
g. All strains of S. pyogenes and most strains of S. porcinus and S. iniae are PYR positive. Other Streptococci are all negative.
h. Nutritional variants Streptococci are now identified in two different genera Abiotrophia and Granulicatella.
i. Unusual strep/genera includes species of Streptococci usually found in animals and Globicatella sanguinis and Dolosicoccus paucivorans.
j. Aerococcus species includes A. viridians, A. urinae, A. sanguicola and A. urinehominis.
k. Salt tolerant Gemella- like bacteria include Alloiococcus, Dolosigranulum, Facklamia and Ignavigranum species.

Table 1: Result of Biochemical Tests of Triclosan (T) Group

S. No	Gas Production	BE Test	Van Test	NaCl Test	45˚C Test	10˚C Test	Inferred Organism
1	–	–	R	–	+	–	Streptococci
2	–	–	R	–	+	–	Streptococci
3	–	–	R	–	+	–	Streptococci
4	–	–	R	–	+	–	Streptococci
5	+	–	R	–	+	+	Leuconostoc
6	–	–	R	–	+	–	Streptococci
7	–	–	R	–	+	–	Streptococci
8	–	–	R	–	+	–	Streptococci
9	–	–	S (38mm)	–	+	–	Streptococci
10	–	–	S (40mm)	–	+	–	Streptococci
11	–	–	S (37mm)	–	+	–	Streptococci
12	–	–	S (30mm)	–	–	–	Streptococci
13	–	–	S (12mm)	+	+	+	Streptococci
14	–	–	S (27mm)	–	+	+	Enterococci
15	–	–	S (25mm)	+	–	–	Streptococci
16	–	–	S (25mm)	+	+	+	Enterococci
17	–	–	S (22mm)	–	+	+	Enterococci
18	–	–	S (25mm)	+	+	+	Enterococci
19	–	–	S (24mm)	+	+	+	Enterococci
20	–	–	R	+	+	+	Enterococci
21	–	+	S (20mm)	+	+	–	Pediococcus
22	–	+	S (15mm)	–	+	–	Pediococcus
23	–	+	S (18mm)	+	+	–	Pediococcus
24	–	–	S (23mm)	–	+	–	Streptococci
25	–	–	S (26mm)	–	+	–	Streptococci
26	–	–	R	–	+	–	Streptococci
27	–	+	S (16mm)	–	+	–	Pediococcus
28	–	–	S (16mm)	–	+	–	Streotococci
29	–	+	S (16mm)	+	+	–	Pediococcus
30	–	–	S (17mm)	–	–	–	Streptococci
31	–	–	S (18mm)	–	–	–	Streptococci
32	–	–	S (18mm)	+	–	–	Streptococci
33	–	–	20mm	+	+	–	Streptococci
34	–	–	20mm	–	+	–	Streptococci
35	–	–	R	–	+	–	Streptococci

Table 2: Result of Biochemical Tests of Triclosan Free (Tf) Group.

S. No.	Gas production	BE Test	Van Test	NaCl Test	45˚C Test	10˚C Test	Inferred column
36	–	+	R	–	+	–	Pediococcus
37	–	+	R	+	+	–	Pediococcus
38	–	–	R	–	+	+	Enterococci
39	–	+	R	+	+	–	Pediococcus
40	–	+	S (28mm)	–	+	–	Pediococcus
41	–	+	S (30mm)	–	+	–	Pediococcus
42	–	–	S (30mm)	–	+	–	Streptococci
43	+	–	R	–	+	–	Leuconostoc
44	–	+	R	–	–	–	Pediococcus
45	+	–	R	–	+	–	Leuconostoc
46	+	+	R	–	+	–	Leuconostoc
47	–	+	S (24mm)	+	+	–	Pediococcus
48	–	+	S (18mm)	+	–	–	Pediococcus
49	–	–	S (20mm)	–	+	–	Streptococci
50	–	–	S (19mm)	–	+	–	Streptococci
51	–	+	S (18mm)	+	+	–	Pediococcus
52	–	–	S (18mm)	+	+	–	Streptococci
53	–	–	S (18mm)	+	+	–	Streptococci

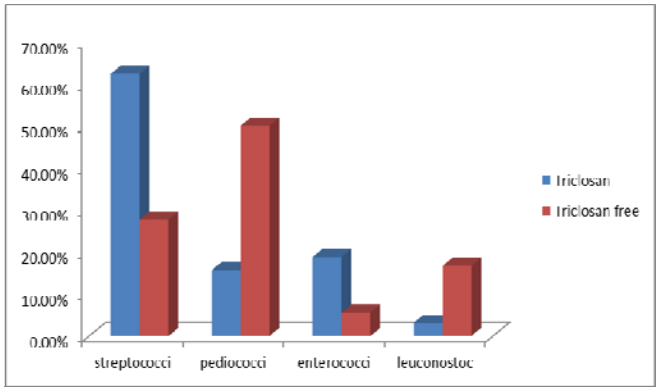
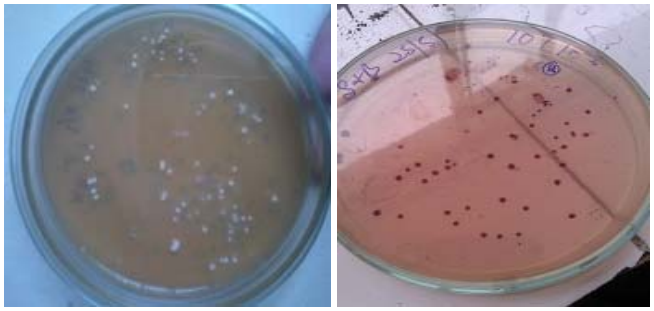


Fig 1: Percentage of culturable genera isolated from both triclosan containing and triclosan free groups.



(A) **(B)**

Fig 2: Diversity of colonies obtained after spreading 100 µl of thioglycolate broth incubated with saliva sample onto (A) Pfizer agar and (B) SNB agar.

(A) **(B)**

Fig 3: Streak-plating of mixed colonies to isolate pure colonies onto (A) Pfizer agar and (B) SNB agar

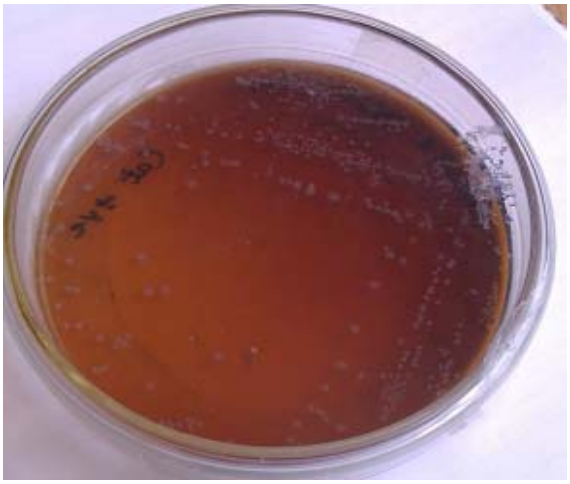


Fig 4: Black color colonies on Pfizer plate

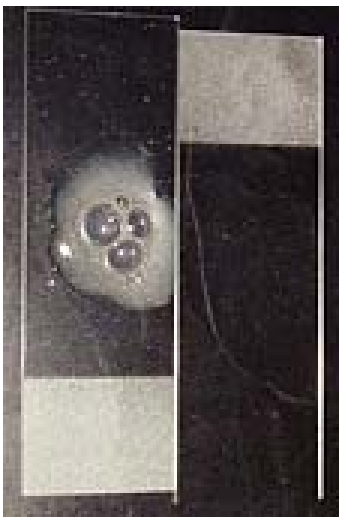


Fig 5: Slides showing positive and negative Catalase test.

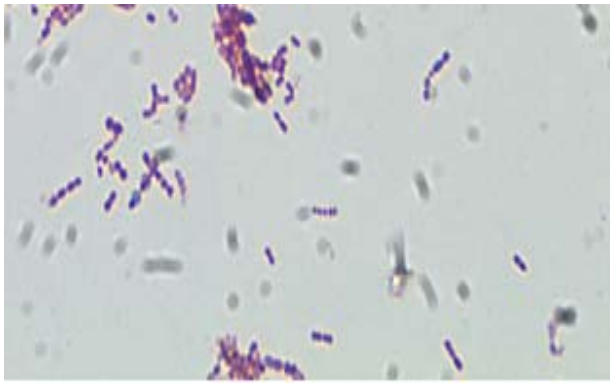


Fig 5: Gram stained isolate Ref. no. 6 (identified as *Streptococci*) [Magnification: 1000X]

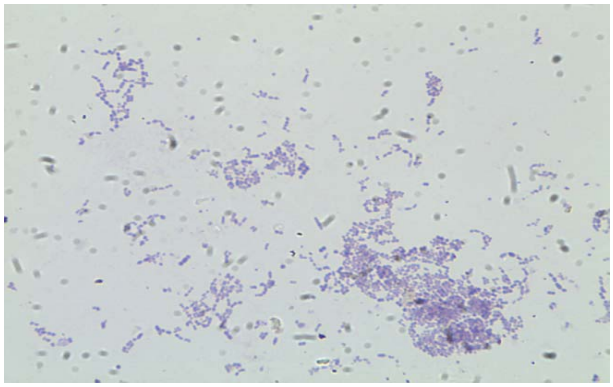


Fig 6: Gram stained isolate Ref. no. 11 (identified as *ENTEROCOCCI*) [Magnification: 1000X]

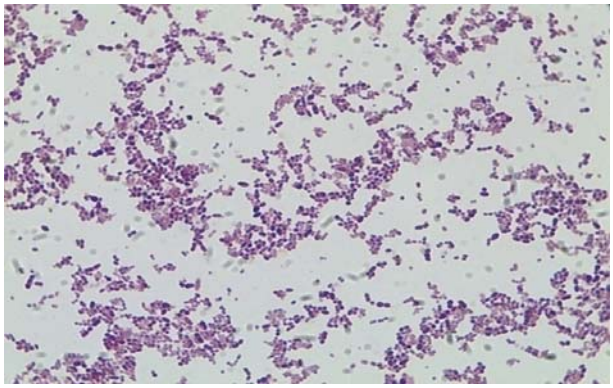


Fig 7: Gram stained isolate Ref. no. 15 (identified as *PEDIOCOCCI*) [Magnification: 1000X]

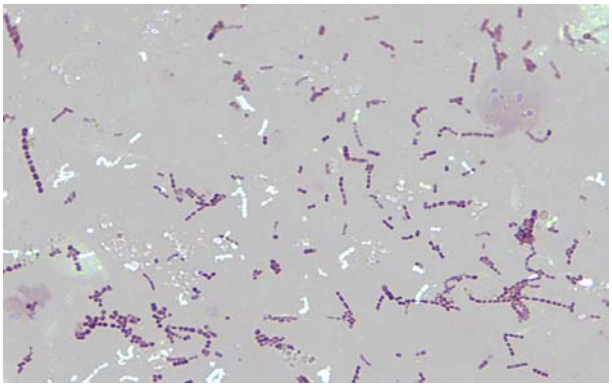


Fig 8: Gram stained isolate Ref. no.9 (identified as *LEUCONOSTOC*) [Magnification: 1000X]

4. Conclusion

This work gives a radically new insight into diversity of human oral microflora. Different microflora from triclosan containing and triclosan free toothpaste user's saliva isolated and characterized. Isolated microflora belongs to Streptococci, Pediococci, Enterococci and Leuconostoc. Percentage of Streptococci and Enterococci is high in Triclosan containing tooth paste user samples. Percentage of Pediococci and Leuconostoc is high in Triclosan free tooth paste user samples.

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