

# Study of Comparison of Oral Fungal Flora of Smokers vs Non-Smokers in a Metropolitan City

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## Abstract

There are some common fungi found in the oral cavity. Epidemiological studies indicate higher risk for periodontal disease in smokers vis a vis non-smokers. The increased risk is proportional to the length and frequency of smoking. This is an important area of public health research. In our study we found fungi to be present significantly more in oral cavity of smokers as compared to non-smokers.

**Keywords:** Oral Microbiome, Pathogenic microorganisms, Smokers, Non smokers

**Abbreviations:** CSC: Cigarette smoke condensate

## 1. Introduction

Cigarette smoking is injurious to health, there are various types of microbes present in our oral flora, some are beneficial some are harmful, but when we smoke or chew tobacco these beneficial microbes start declining and pathogenic microbes start multiplying their number, which leads to different types of oral problems. Cigarette smoke concentrate (CSC) comprises in excess of 7000 known chemical molecules of which 6 are complex mixture of oxidants and various free radicals. It can modify the oral microenvironment and lead to significant oxidative stress, which in turn can damage the oral epithelial barrier.<sup>1</sup>

Cigarette smoking is a cause to many abnormal alterations that can begin with an increase of *candida* colony count that causes oral diseases such as pseudomembranous candidiasis, acute atopic candidiasis, chronic hyperplastic candidiasis, chronic atopic candidiasis, and angular cheilitis<sup>9</sup>. Other factors such as long-term antibiotic consumption, HIV infection, diabetes mellitus, radiotherapy, bad oral hygiene, dental prostheses, and cigarette smoking can also contribute to an increase of candida colony count<sup>[9,10,11]</sup>.

## 2. Materials and Methods

### 2.1. Timeline of study

This study was carried out in a span of 3 months from February 2023 to May 2023.

### 2.2. Place of study

Department of Microbiology, Bidhan Nagar campus, All India Institute of Hygiene and Public Health, Kolkata.

### 2.3 Type of study

Laboratory based observational study.

### 2.4 Sample size

Fifty four (54) subjects, representing both genders, ranging in age from 18 to 60. Fifty-four (54) samples were tested. This sample size had been calculated by method of convenience. In case of smokers the swabs were taken from those, who smoked from a minimum of 5 years. There were 27 swabs taken from smokers and 27 swabs from non-smokers. Two swabs were taken from each volunteer, one for culture and another for Gram stain and Albert's stain. Gram stain for seeing gram positive and gram negative bacteria while Albert stain for seeing bacteria containing Metachromatic Granules(MCG)16,17.

### 2.5 Methodology proper

These volunteers were from the below-mentioned campuses or worked there:-

- a) Bidhannagar campus
- b) Urban health unit and Training centre, All India Institute of Hygiene and Public health, Chetla
- c) Central Avenue (main campus),
- d) Sonarpur
- e) Howrah

Samples from the following nine sites were analyzed for each subject: dorsum of the tongue, lateral sides of the tongue, buccal fold, hard palate, soft palate, labial gingiva and tonsils of soft tissue surfaces, and supragingival and subgingival plaques from tooth surfaces. samples were be collected by swab from the adjacent area and was brought to the laboratory. Then samples were transported to the laboratory in an ice-pack or within 4 hours of collection. Then the samples were processed for bacteria and Yeast. Samples were inoculated on the following media:

1. Mac Conkey agar with neutral red as pH indicator (Peptone, Neutral red, agar agar, Lactose, Sodium taurocholate, deionized water) for distinguish bacteria based on their lactose-fermenting properties into LF (Lactose-fermenting) and NLF (Non-Lactose-fermenting) colonies..
2. Sabouraud's dextrose agar (SDA) plate (pH 5.6-6) (containing D-glucose 2gm, Peptone 2 grams, Agar agar 2 grams, and deionized water 100 ml) for fungal isolation.
3. Robertson's cooked meat medium (RCM) for culturing anaerobes, made as per manufacturer's instruction .
4. Blood agar plate for differentiating bacteria based on their pattern of hemolysis.
5. Muller Hilton Agar for Antibiotic Susceptibility Test.
6. Nutrient Agar with 6.5% NaCl and Tellurite Agar for inoculation of Enterococcus spp.
7. Egg Yolk Agar for inoculation of Bacillus spp.

Gram's stain and Albert's stain were carried out from samples directly, to detect Gram positive or gram negative bacteria, and bacteria with metachromatic granules respectively, as another Add the fungal colony to the drop of LPCB using a sterile mounter an inoculation loop (from solid medium), depending on the sample of use part of the study.

Samples were inoculated on specific media and identification of microorganisms were done.

### Identification of fungus

*A: LPCB mount showed budding yeast:*

Lactophenol Cotton Blue (LPCB) Staining is an easy method employed for the microscopic examination and subsequent identification of fungi. This method works on the principle of aiding the identification of the fungal cell walls.

1. On a clean & sterile microscopic glass slide, we added a drop of LPCB solution.
2. We added fungal colony on the drop of LPCB solution, using a sterile mounter or inoculation loop (from solid medium), depending on the sample of use.

3. Tease the fungal sample of the alcohol using a needle mounter, to ensure the sample mixes well with the alcohol.
4. Carefully cover the stain with a clean sterile coverslip without making air bubbles to the stain.
5. Examine the stain microscopically at 40X, to observe for fungal spores and other fungal structures.

*B. Germ tube test:*

Two to three colonies of yeasts were passed in 0.5 ml of pooled human serum in a small test tube and incubated aerobically at 37°C for 2-4 hours. Then a wet mount was prepared from the suspension and observed under the 10X and 40X objectives of the light microscope.

Positive germ tube was declared when there was tube-like narrow elongated projection without constriction at base.

Germ tube positivity is found in *Candida albicans* and *Candida dubliniensis*.

*C. Sugar fermentation test:*

Sugar fermentation test was done in peptone water containing 2%(w/v) sugar (Lactose, Sucrose, Glucose , Maltose) and Andrade Indicator (contains Acid Fuchsin and NaOH). Colour change and pellicle formation were noted and interpreted as follows:

*Candida albicans*:- Glucose and Maltose fermented, sucrose variable, lactose not fermented.

*Candida tropicalis*:- Glucose , Maltose and sucrose fermented, lactose not fermented.

*Candida glabrata*:- Glucose fermented, Maltose, lactose and sucrose not fermented.

*Candida kefyr*:- Glucose, sucrose and lactose fermented, lactose not fermented.

Sugar fermentation test results are shown in figure 1 below.



**Fig1:** Sugar Fermentation Test

*D. Thermotolerance test:*

Germ tube test positive *Candida* isolates were inoculated on SDA tube and kept at 44.5°C in the Water bath overnight. If growth shown in SDA tube the next day, then the isolate interpreted as *Candida albicans*, growth not shown in SDA tube then it was noted as *Candida*.

*E. Dalmau test:*

It was done on Corn Meal Agar. With the help of loop or straight wire, yeast colonies were taken, slit culture was done on the Corn meal Agar. It was incubated at room temperature for 2-3 days in a dark place. After 2-3 days of time, the inoculated Corn meal agar plate was observed under the 10X and 40X objectives of the compound microscope. If terminal chlamydo spores were observed under the microscope then a confirmation test will be done. Terminal chlamydo spores are seen by Dalmau test in *Candida albicans* as well as *Candida dubliniensis*. Dalmau test is shown in figure 2 below.



**Fig 2:** Dalmau Test.

Scheme for yeast Identification (G= glucose, M=Maltose, S+ Sucrose, L= lactose) is shown in Table 1 below.

**Table 1:** Results of Dalmau, Germ tube test and sugar fermentation of yeasts.

Sl. No	Name of yeast	Colony character	Germ tube test	Sugar fermentation	Thermotolerance test at	Dalmau test
1	<i>Candida albicans</i>	Moist pasty colonies on SDA.	Positive	G+ M+ S (Variable) L-	positive	Single Terminal chlamydo spore
2	<i>Candida dubliniensis</i>	Moist pasty colonies on SDA.	Negative	G+, M+, S (VARIABLE), L ,	negative	Multiple Terminal chlamydo spore
3	<i>Wickerhamomyces anomalus</i>	Moist pasty colonies on	Negative	G+, M+, S +, L-,	negative	Only yeasts
4	<i>Candida kefyr</i>	G+, M+, S (VARIABLE), L-,	Negative	G+, M-, S +, L+,	negative	Logs in stream appearance of yeasts and pseudo-hyphae

We did a biofilm formation test for every *C.albicans* isolate by test tube method using 1% alcoholic Safranin.

### 3. Results

#### 3.1 How many samples tested

In this study 54 samples were collected, out of which 14 were taken from females and 40 from males. Out of 14 females, 7 were smokers and 7 were non-smokers. Out of 40 males 20 were smokers and 20 were non-smokers. Out of 27 smokers 7 were occasional smokers while 20 were regular/chain smokers. Of all the subjects who were smokers, only 1 smoked bidi(handmade smoking stick) while others smoked cigarettes. Out of all the smokers, 4 individuals were also consuming oral tobacco.

The Biofilm formation test on every *C.albicans* isolation was positive.

The Fungal species we isolated in smokers was *Candida albicans* (14 out of 27) (51.85%), *Candida dubliniensis* (4 out of 27) (14.81%), *Wickerhamomyces anomalous* (5 out of 27) (18.51%), *Candida kefyr* (2 out of 27) (7.40%) and *Trichosporon* (2 out of 27) (7.40%). *Candida albicans* was the commonest fungus isolated from smokers.

We isolated no fungal species in Non smokers. (sample size of non-smokers was 27).

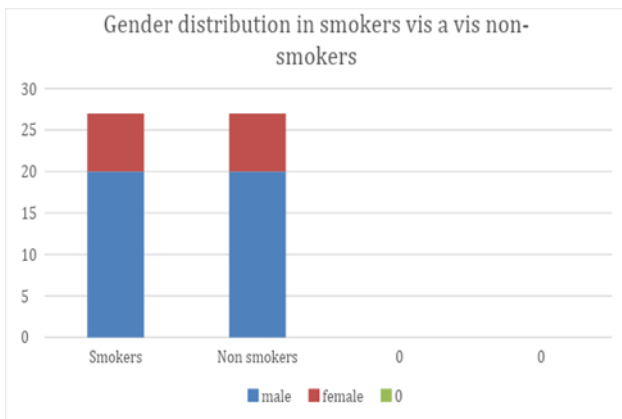


Fig 3: Gender distribution of Smokers vs nonsmokers

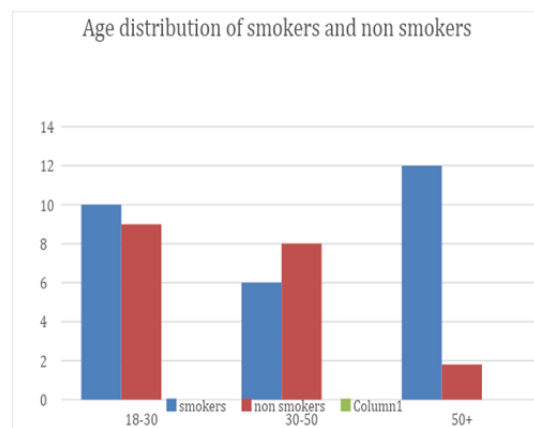


Fig 4: Age distribution of Smokers vs nonsmokers.

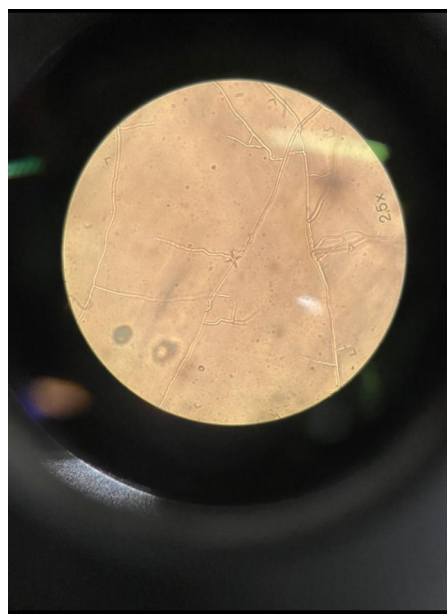


Fig 5: Microscopic image of *Trichosporon* spp.

All the *Candida* isolates were susceptible to Fluconazole in vitro, by the disk diffusion test on Mueller Hinton Agar containing 2 gm/ 100 ml Glucose and 0.5 micrograms per ml Methylene blue.

## 4. Discussion

Smoking is one of the major factors affecting the pulmonary, cardiovascular and some infectious diseases. Smoking can influence both the innate and adaptive immune responses, both humoral and cell mediated immunity.<sup>1</sup>

In the present study we collected samples from male and females between the age range of 18-60 years. Though our sample size of Smokers and Non smokers was 54.

In this study we isolated fungus in smokers only. The most prevalent species was *Candida albicans*.<sup>2</sup>

The trend of cigarette smoking is on the rise in developing countries like India. Youth are attracted towards it, which is an alarming sign.<sup>3</sup>

Nicotine and other particles in tobacco smoke increase *C.albicans* adhesion and also biofilm formation<sup>4</sup>.

One limitation of our study was that the sample size was small (54).

The present study can infer that smoking cigarettes can increase the risk of candidiasis. We also found *Candida* spp. in smokers. However, this study was carried out in a restricted geographical setting with a limited sample size. The habit of smoking is recognized as a very important predisposing factor for Oral candidiasis, because it can incite more keratinization in the oral epithelial cells. In addition to this, the smoke constituents increase fungal virulence. Many studies have documented that smoking can stimulate increase in carriage of oral candidiasis<sup>5</sup>. More such studies should be done in future for the sake of public health<sup>6</sup>.

CSC (Cigarette smoke concentrate)- mediated induction of *C. albicans* adherence to cells, growth, and also biofilm formation may explain the increased persistence of this pathogen in smokers. These findings may also be relevant in case of other oral diseases where biofilms are found.<sup>7</sup> Exposure to cigarette smoke increases fungal growth and biofilm production. Cigarette smoking interferes between *S mutans* and *C.albicans* results in biofilm formation on dental restoration materials<sup>7</sup>. Cigarette smoke reaches not only the host cells but also the microorganisms of the oral cavity. More contact between cigarette smoke and the oral microbes can enhance development of diseases like periodontitis. Cigarette smoke may also fine-tune phenotypic and other traits of *C. albicans* which promote oral candidiasis<sup>8</sup>. Smoking can affect oral colonization due to *Candida* species. Both overall have a bad effect on oral health<sup>9</sup>. Oral *Candida* infection is usually seen in an immunocompromised host and the compromise may be either local or systemic. Local compromising factors like diminished salivation, poor oral hygiene, wearing dentures, and other systemic factors like Diabetes mellitus, nutritional deficiencies, HIV infection and others also promote oral infections like oral Candidiasis<sup>10</sup>.

## Conclusion

Exposure to cigarette smoke enhances fungal growth and biofilm formation. More studies are needed in this aspect.

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