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# The tongue microbiome in healthy subjects and patients with oral diseases: The first study in Algeria

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**ABSTRACT**

**Background:**The tongue microbiome represents an important reservoir of pathogens, indicating an established risk for infections.The purpose of this study was to identify the tongue microbiota in subjects with and without oral diseases and compare oral health status between sexes. **Methods:** The sample consisted of 92 subjects, divided into the following groups: 17 xerostomia patients, 21 periodontitis patients, 22 gingivitis patients, 14 patients with caries, and 18 control participants. Samples were collected from the dorsal and ventral surfaces of the tongue for each subject. Microbial strains were identified using API and Vitek2. **Results:** There were no statistically significant differences in the detection of microorganisms between the two surfaces of the tongue ($p$= 0.706).The major isolated bacterial species from the tongue samples was *Staphylococcus aureus* (13% in xerostomia, 13% in gingivitis, 16% in periodontitis, 18% in caries and 24% in healthy subjects). While the most common fungal species was *Candida albicans* (39% in xerostomia,36% in gingivitis,18% in periodontitis, 25% in caries and 21% in healthy subjects).The distribution of yeast and bacteria varied between groups for certain species but was similar for other species. Moreover, there was a difference in susceptibility to oral diseases between males and females ($p$= 0.014). **Conclusions:** This study confirms the richness of the tongue in a variety of bacteria and fungi species in all groups and highlights the difference in susceptibility to oral diseases between sexes.

## Introduction

The microbial community inhabiting our bodies, the microbiome [1], comprises a core microbiome shared by all healthy people and a variable microbiome that changes between persons based on their phenotypic and genotypic markers as well their habits [2,3].The oral cavity contains the second largest microbiome in the body [4] , consisting of over 700 bacterial, fungal, archaeal and viral species [5]. Each oral site, including the tongue, and teeth, has unique characteristics, creating different environments for microbial proliferation [6]. The tongue microbiome is more diverse and denser in comparison to other mucosal tissues's microbiomes [7,8]. The tongue's dorsal surface is predominantly colonised by bacterial genera including *Streptococcus*, *Veillonella*, *Prevotella*, *Actinomyces*, *Fusobacterium*, *Neisseria* [9], *Leptotrichia* [10], *Staphylococcus*, *Haemophilus*, *Klebsiella*, *Acinetobacter*, *Enterobacter*, *Corynebacterium*, *Pseudomonas*, *Bacillus*, *Proteus* and *Rothia* [11,12]. The tongue's ventral surface is colonised by *Streptococcus*

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species (spp.), *Neisseria* spp., *Aggregatibacter* spp., *Eikenella* spp., *Eubacterium* spp., *Gemella* spp., *Treponema* spp. [13], *Staphylococcu*s spp., enterobacteriaceae spp. [14, 15], and *Leptotrichia* spp. [10]. Tongue's surfaces are colonised by fungi like *Candida* spp., *Epicoccum* spp., *Cladosporium* spp., *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., *Cryptococcus* spp., *Aureobasidium* spp. [16, 17], *Malassezia* spp., *Irpex* spp., *Cytospora* spp., *Sporobolomyces* spp. [17], *Saccharomyces* spp., *Rhodotorula* spp. [18], and *Trichosporon* spp. [19]. Tongue microorganisms live in balance [20], inhibiting pathogen colonization, through the limitation of pathogen binding sites, competition for nutrients, creation of unfavorable environments, and production of antimicrobial peptides [21]. Microbial imbalance, resulting from immunosuppression, altered salivary function [22], medication use, dietary behaviors, and poor oral hygiene [23], causes periodontal diseases [24] and caries [25]. In periodontal diseases, the microbiome shifts from beneficial to pathogenic bacteria, causing inflammation and tissue degradation, through proteinases release that degrade periodontal structures, toxins production that incite host cells to produce degradative enzymes, or stimulation of immune response to release proinflammatory cytokines [26]. An overgrowth of acid-producing bacteria, which metabolize carbohydrates into organic acids, reduce the pH below normal range [27], causing tooth enamel demineralization and leading to caries [28]. Fungi are secondary contributors to oral diseases by their co-aggregation with bacteria, which stabilizes pathogenic microbiome [29].

Approximately half of the world's population experiences oral disorders [30], which can cause disfigurement, pain, and mortality. These conditions are a serious public health problem in many countries [31].

The disparities between sexes in oral health are influenced by social, behavioral, and biological factors [32]. Males are more susceptible to periodontitis [33], while females are more prone to gingivitis [34], caries [35], and xerostomia [36].

Periodontal pathogens can destroy the immunity, induce inflammatory cytokine production, and enter the bloodstream through damaged periodontal tissue, causing systemic inflammation and systemic diseases [37- 39]. Oral microbiota is linked to insulin resistance by influencing immune inflammation, resulting in diabetes development [40], respiratory diseases result from oral microbiota inhalation into the lungs [41].

Previous studies have predominantly focused on the tongue dorsum microbiome, leaving the microbiome of the tongue's ventral surface poorly understood, particularly in relation to health and disease. There is no knowledge regarding the tongue microbiome of Algerian individuals. This study aimed to identify and compare the composition of microbiome on the dorsal and ventral tongue's surfaces, to examine sex-based differences in susceptibility to oral diseases, and to determine the microbiology associated with oral health and oral diseases.

## Materials and methods

### Subjects'population

A total of 92 subjects aged 11 to 77 years were included in this study. Of these, 18% suffered from xerostomia, 24% from gingivitis, 23% from periodontitis, 15% from dental caries, and 20% were free of any oral disease (control group). The participants were recruited from the dental clinic of Dr. XXXX University Hospital Center in Tlemcen, Algeria (from September 2021 to June 2022). All participants were requested to complete a questionnaire and were examined by a qualified dentist for the diagnosis of oral diseases. The exclusion criteria were children under 10 years old and unwilling participants in the study.

### Ethical consideration

The study protocol was conducted in accordance with the tenets of the Helsinki Declaration and was approved by the Human Research Ethics Committee of Tlemcen University. All participants, or their designated representative, provided informed consent.

### Samples collection

The samples were obtained from two distinct sites on the tongue of each subject: one from the dorsal surface and the other from the ventral surface, using sterile swabs. Each specimen was promptly transferred to a sterile tube for transportation to the laboratory.

### Microbial analysis of the samples

A volume of 3 ml of physiological water (PW) was added to each specimen, which was then vortexed for 2 minutes. Subsequently, 200 µL of this suspension was taken and distributed equally

between two tubes. To the first tube, 900 µL of nutrient broth was added to study bacteria, while 900 µL of Sabouraud dextrose broth (SDB) was added to the second tube to study yeast. The samples were then incubated for 24 to 72 hours at 37°C for bacterial growth and at 35°C for fungal growth. The PW remaining in the initial sampling tube was replaced with SDB. These tubes were then incubated for 24 to 72 hours at 35°C [42]. Following the incubation of the specimens, a series of different culture media were employed to isolate the strains. These included Mannitol Salt Agar for the isolation of *Staphylococcus* spp., MacConkey agar for the isolation of enterobacteria, Cetrimide agar for the isolation and differentiation of *Pseudomonas aeruginosa*, Sabouraud dextrose agar for the isolation of yeast, and Chromagar Candida for the differentiation of *Candida* spp. The plates were then incubated aerobically at 37°C for 24-48 hours to allow for the bacterial growth, and at 35°C for 24-72 hours to allow for the fungal growth. Isolated strains were identified using API (Biomerieux) and the VITEK® 2 automated identification system.

### Statistical analysis

The data was analyzed using IBM Statistical Product and Service Solutions (SPSS) version 26.0. A chi-square test ($\chi^2$) was employed to extract the oral health differences between sexes, to compare the composition of the microbiome on the dorsal and ventral tongue's surfaces, while ANOVA test was employed to compare the number of microorganisms isolated from patients and control groups. The threshold for statistical significance was set at $p< 0.05$. The sample size calculation and the generation of graphs were conducted using Microsoft Office Excel 2007.

## Results

### Socio-demographic characteristics of study participants

This study included a sample of 92 patients. The sample size was 75 and the mean age of the study participants was 34.90 ± 15.56 years. The majority of them were female (57%), with males comprising 43% of the sample **(Table 1)**.

### Distribution of oral diseases by sex

A significant difference was identified between the oral health profiles of females and males ($p= 0.014$). Females were more susceptible to xerostomia, gingivitis, and caries, while males were more prone to periodontitis **(Table 2)**.

### Oral hygiene practices in population study

### Tongue brushing

**Figure 1** demonstrated the percentages of study participants who self-reported tongue-brushing. Most individuals in the five groups did not practice tongue-brushing. The highest percentage of non-practice was observed in the xerostomia group (82%).

### Tooth-brushing

**Figure 2** showed the proportion of study subjects who self-reported tooth-brushing habits. Among the control, periodontitis, xerostomia, and gingivitis groups, the highest proportion of individuals reported tooth-brushing twice a day (44%, 38%, 35%, and 27%, respectively). The highest proportion in caries patients reported brushing their teeth once a day (36%).

### Dietary habits

The data indicated that 72% of the study population consumed higher amounts of sugar, with the following breakdown: 11% of caries patients, 14% of periodontitis patients, 14% of gingivitis patients, 16% of xerostomia patients, and 17% of control subjects.

### Identification of microorganisms in tongue samples

### At the family level

There were no significant differences in the presence of microbial families between the dorsal and ventral tongue's surfaces ($p= 0.706$) **(Figure 3)**.

Staphylococcaceae was the dominant bacterial family on both tongue's surfaces. In contrast, *Enterobacteriaceae*, Enterococcaceae, Aerococcaceae, Micrococcaceae, Streptococcaceae, Moraxellaceae, and Pseudomonadaceae were found in low percentages **(Figure 3)**.

Cryptococcaceae was the major fungal family on both tongue's surfaces. However, the presence of Saccharomycetaceae and Trichosporonaceae families was low **(Figure 3)**.

### At the species level

### Bacterial species

*Staphylococcus aureus* was the dominant member of the Staphylococcaceae family. In the Enterobacteriaceae family, it was found that *Klebsiella oxytoca* and *Klebsiella pneumoniae* ssp. *pneumoniae*. were more isolated than other species. The *Enterococcaceae* family was represented by two species, *Enterococcus faecalis* and

*Enterococcus faecium*. The isolation percentages for species belonging to other bacterial families were low.

**Fungal species**

The *Candida* genus was the most dominant in the Cryptococcaceae family, with *Candida albicans* representing the predominant species. Two samples yielded the presence of *Saccharomyces cerevisiae*, while *Trichosporon asahii* was isolated on two occasions.

**Microbiology of oral diseases**

**Xerostomia patients**

**Figure 4** showed the bacterial strains that are isolated from both tongue's surfaces in xerostomia patients. The most common strain was *S. aureus* (13%), followed by *Staphylococcus hominis* (9%), *Staphylococcus (Staph.) epidermidis* (7%), and other species.

**Figure 5** showed the fungal strains that isolated from both tongue's surfaces in xerostomia group. The most common fungal strain was *Candida albicans* (39%), followed by *Candida kefyr* (5%), *Candida famata* (5%), and other species.

A total of 41% of xerostomia patients suffered from allergy, hypertension (HTA), diabetes mellitus (DM), bilateral carotid artery stenosis, thyroid disorders (TD), and sinusitis.

**Gingivitis patients**

**Figure4** showed the bacterial strains that isolated from both tongue's surfaces in gingivitis patients. The most common strain was *S. aureus* (13%) followed by *Staph. haemolyticus* (8%), *Staph. epidermidis* (8%), and other species.

**Figure 5** showed the fungal strains that are isolated from both tongue's surfaces in gingivitis group. The most common strain was *C. albicans* (36%) followed by *Candida (C.)famata* (5%), *and C. parapsilosis* (2%).

A total of 41% of gingivitis patients suffered from allergies, migraines, HTA, TD, and rheumatism.

**Periodontitis patients**

**Figure 4** showed the bacterial strains that are isolated from both tongue's surfaces in periodontitis patients. The most common strain was *Staph. aureus* (16%) followed by *Staph. epidermidis* (12%), and other species.

**Figure 5** showed the fungal strains that are isolated from both tongue's surfaces in periodontitis group. The most common strain was *C. albicans* (18%) followed by *C. famata* (6%), *C. kefyr* (4%), and other species.

A total of 29% of periodontitis individuals suffered from hypercholesterolemia, sinusitis, TD, HTA, DM, and heart murmur.

**Caries patients**

**Figure 4** showed the bacterial strains that isolated from both tongue's surfaces in caries patients. The most common strain was *Staph. aureus* (18%) followed by *Staph.epidermidis* (9%), *Staph. warneri* (6%), and other species.

**Figure 5** showed the fungal strains that isolated from both tongue's surfaces in caries group. The most common strain was *C. albicans* (25%) followed by *C. tropicalis* (6%), and other species.

A total of 36% of caries patients suffered from allergies, DM, cancer, and asthma.

**Healthy subjects**

**Figure 4** showed the bacterial strains that are isolated from both tongue's surfaces in healthy subjects. The most common strain was *S. aureus* (24%), followed by *E. faecalis* (8%), *S. hominis* (8%), and other species.

**Figure 5** showed the fungal strains that are isolated from both tongue's surfaces in control group. The most common strain was *C. albicans* (21%), followed by other species such as *C. dubliniensis*, and *C. krusei.*

**Table 1.** Participant characteristics.

| Participant characteristics | n=92 (%) |
|---|---|
| **Gender** | |
| Female | 53 (57) |
| Male | 39 (43) |
| **Age group** | |
| 11-20 | 17 (18) |
| 21-30 | 29 (32) |
| 31-40 | 18 (20) |
| 41-50 | 12 (13) |
| 51-60 | 08 (09) |
| 61-70 | 05 (05) |
| 71-80 | 03 (03) |

**Table 2.** The oral diseases reported by patients according to sex.

| Oral diseases | Female n= 53(%) | Male n= 39(%) | *P* value |
|---|---|---|---|
| Xerostomia | 12 (23) | 05 (13) | |
| Gingivitis | 15 (28) | 07(18) | |
| Periodontitis | 02 (04) | 19 (49) | 0.014 |
| Dental caries | 10 (19) | 04 (10) | |

**Figure 1.** Tongue-brushing percentages in Algerian subjects.

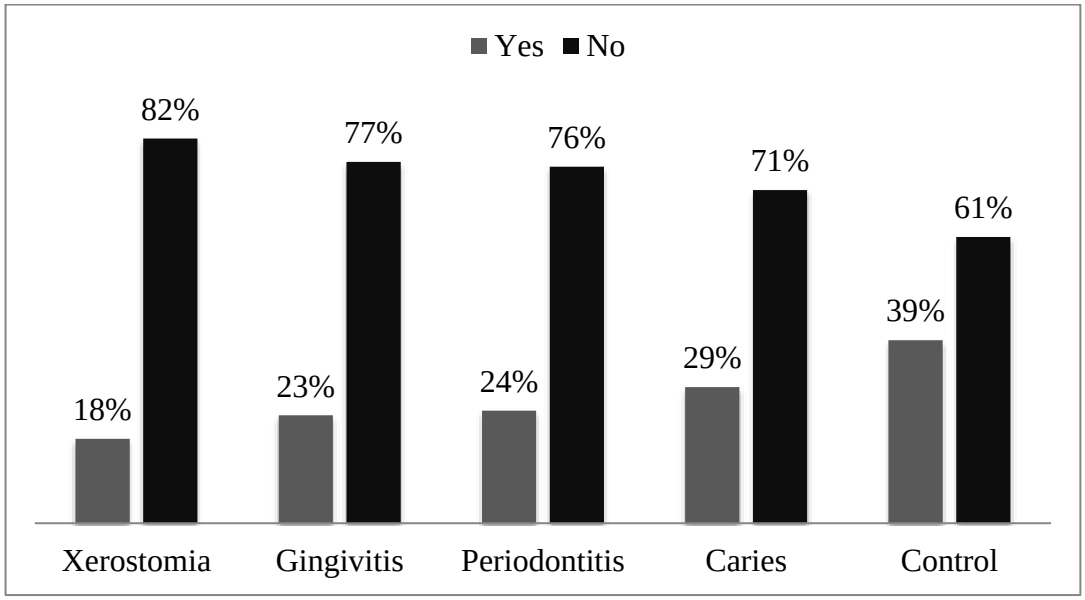


**Figure 2.** Tooth-brushing percentages in Algerian subjects.

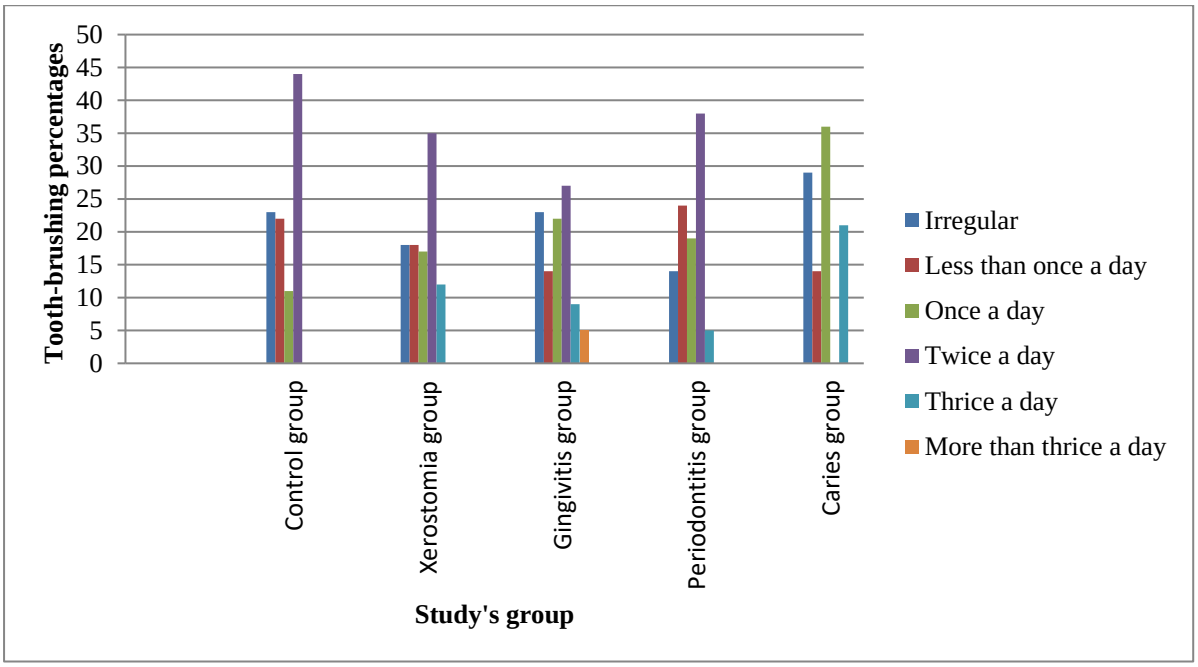



**Figure 3.** Presence of different microbial families in samples of the tongue

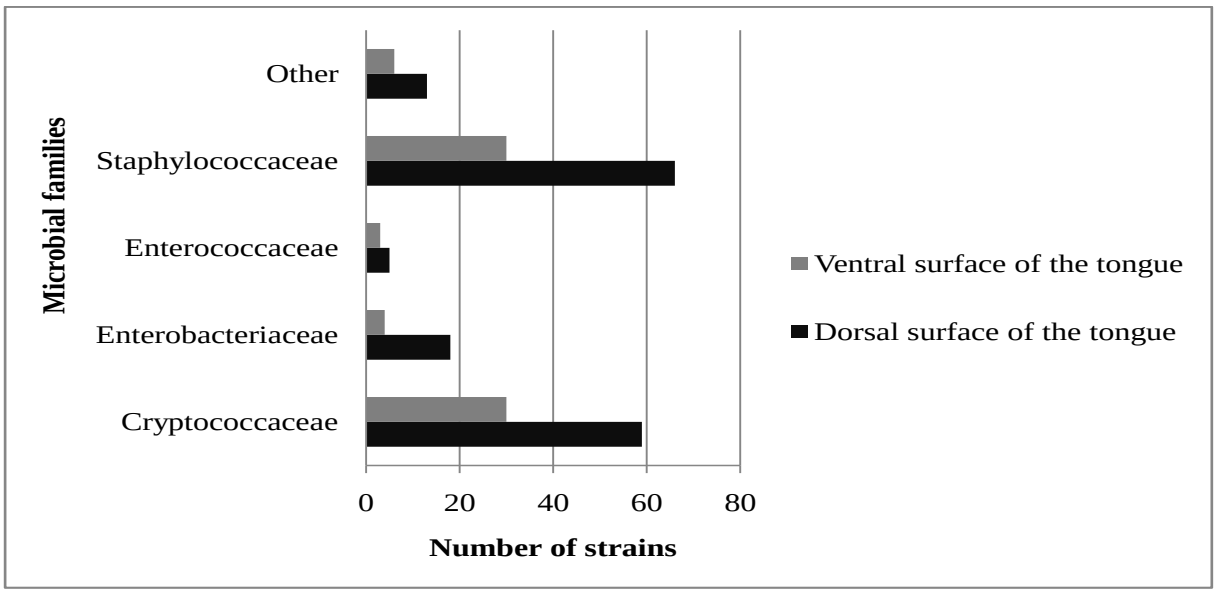
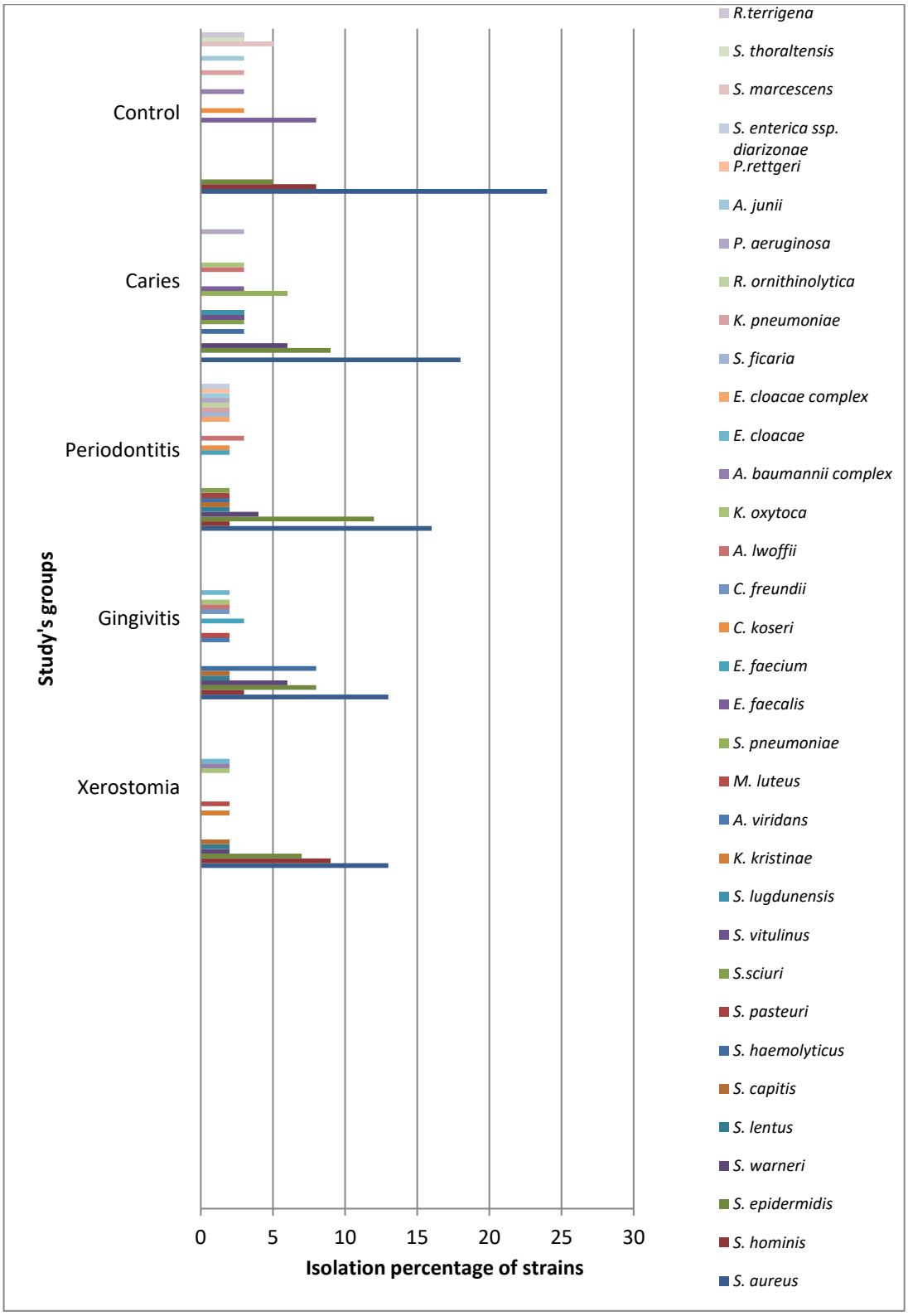
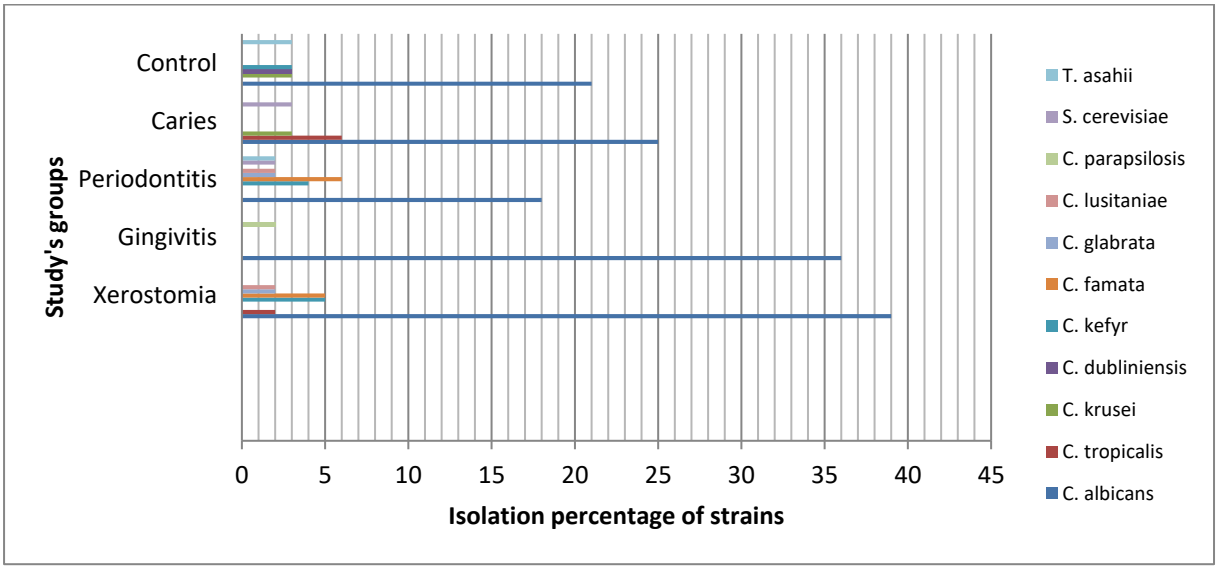


**Figure 4.** Distribution of bacterial species in study's group.




S: *Staphylococcus*, R: *Raoultella*, C: *Citrobacter*, M: *Micrococcus*, *A. viridians*: *Aerococcus viridians*, K. *Kristinae*: *Kocuria Kristinae*, E: *Enterococcus*, *S. pneumoniae*: *Streptococcus pneumoniae*, A: *Acinetobacter*, K: *Klebsiella*, *E. cloacae*: *Enterobactercloacae*, S. *ficaria*: *Serratia ficaria*, *P. rettgeri*: *Providencia rettgeri*, *S. marcescens: Serratia marcescens*, *S. enterica ssp. Diarizonae: Salmonella enterica ssp. Diarizonae*, *S. thoraltensis : Streptococcus thoraltensis*, *P. aeruginosa : Pseudomonas aeruginosa*

**Figure 5.** Distribution of fungal species in study's group.




*C: Candida, S: Saccharomyces, T: Trichosporon*

**Discussion**

This study is the first to characterize the tongue's microbial flora of Algerian individuals, both with and without oral disease, using culture-based methods.A considerable variety of bacteria and fungi were identified.

No significant differences were found in the presence of bacteria and fungi between the tongue's surfaces ($p$= 0.706), consistent with findings from US [43]. However, studies in the UK [44], China, and India [45] yielded opposing results. The microbial composition on the tongue may differ between its surfaces. The rough dorsal surface offers more space for microbial adhesion, while the smooth ventral surface provides a more stable microenvironment. The composition may remain similar due to microbial migration between the surfaces, influenced by their proximity and shared exposure to saliva and nutrients.

*Staphylococcus aureus* was the dominant bacterium colonising tongue's surfaces. Different dominant species were detected: *Streptococcus salivarius* in Japan [46] and Sweden [47], and *Rothia mucilaginosa* in the US [48]. The tongue microbiome varies between populations due to host genetics [49], immune responses [50], and environmental factors [51]. Genetic differences in immune-related genes including those encoding toll-like receptors, influence the microbial recognition and inflammatory responses, impacting microbial adhesion and colonisation [49]. Variations in salivary proteins, encompassing antimicrobial peptides, can influence microbial proliferation [52]. Oral hygiene [53], diet [54], smoking [55], and climate [51] also modify the microbiome.

*Candida albicans* is the dominant fungus on the tongues of Brazilian [56], Chinese [57], and New Zealand [58] populations, consistent with our findings. Individuals from Iraq [59] and the US [60] exhibited higher prevalence of *Aspergillus* spp. and *Malassezia* spp., respectively.

Xerostomia is more prevalent in females than males [36], as observed in India [61], Iran [62], Iraq [63], US [64], Saudi Arabia [65], and Sweden [66], consistent with our findings. Females exhibited greater medication use [36], estrogen deficiency following menopause [67], higher susceptibility to Sjögren's syndrome [67], depression [68], and smaller size of salivary glands [69], explaining their predisposition to xerostomia.

Medications may have a deeper impact on xerostomia than the underlying disease itself [70-73]. Among xerostomia patients, 29% of females and 12% of males were taking medications.

Hyposalivation increases *Candida* spp. numbers [74], with *C. albicans* was the most isolated microorganism in xerostomia patients (39%), significantly higher than in the control group (21%) (P=0.013). This aligns with studies from Brazil [69], Japan [75], and India [61]. Reduced saliva production [76], lowered salivary antimicrobial proteins secretion, reduced pH [77],

altered oral mucosal integrity and dysregulated immunity in xerostomia promoting growth and adherence of fungi. Staphylococci were increased in xerostomia individuals from US [78] and Sweden [79] and remained consistent in our study. Acidic pH in xerostomia does not promote staphylococci overgrowth [80, 81].

The prevalence of gingivitis is higher in females than males, consistent with studies in Palestine [34], Pakistan [82], Portugal [83], and Nepal [84]. In China, the prevalence was similar between sexes [85]. Elevated progesterone and estrogen levels during puberty or pregnancy enhance gingivitis [34].

A total of 86% of gingivitis patients have dental calculus, a risk factor for this disease [86]. Dental calculus results from poor oral hygiene and elevated sugar consumption [87].

*Candida albicans* was more isolated from gingivitis patients (36%) than from healthy subjects (21%) (P=0.021), as observed in China [88] and Egypt [89]. *Candida* spp. may associate with bacteria, or stimulate the pro-inflammatory cytokines generation, contributing to gingivitis [90]. The microbiota imbalance in gingivitis stimulates an inflammation, increases cytokine levels, and lowers the oral pH to acidic levels, which promote the *C. albicans* growth [91-93].

*Staphylococcus aureus* was the most isolated Staphylococci in healthy and gingivitis groups, with 24% and 13% of strains, respectively (P=0.505), consistent with studies in Iraq [94] and Greece [95]. In Brazil, this bacterium was less isolated in gingivitis patients [96]. Notwithstanding inflammation, *S. aureus* occurrence is not increase, as it colonises transiently and grows slowly in the low-oxygen environment caused by gingivitis [97]. Biofilm formation and antimicrobial resistance in staphylococci, enhance gingivitis pathogenesis [98].

Periodontitis is more prevalent in males than females [99], as observed in Jordan [100], India [101], Pakistan [82], Portugal [83], and US [102], consistent with our findings. Males exhibited poor oral hygiene [103], higher smoking rates [104], insufficient dental visits [105], lower levels of protective hormones estrogen and progesterone [105], and heightened genetic predilection towards periodontitis [106]. This explains the higher prevalence in males.

There were no significant differences in *C. albicans* isolation between periodontitis (18%) and control (21%) groups ($p$= 0.742), as found in the US [107] and Chile [108]. *Candida* spp. were more prevalent in periodontitis patients than healthy subjects [109]. In periodontitis, pathogenic bacteria outcompete *Candida* for nutrients and space, limiting *Candida's* growth. The more anaerobic environment in periodontitis prevents *Candida* overgrowth, unlike in gingivitis.

*Staphylococcus aureus* was the most isolated Staphylococci in both groups, with no significant difference between them ($p$= 0.643), as observed in Greece [95], this specie was less prevalent in periodontitis patients than non-affected individuals in Brazil [96]. *Staphylococcus epidermidis* was the predominant isolated staphylococci in periodontitis patients in Brazil [110] and in UK [111]. In this study, coagulase-negative staphylococci (CoNS) were more isolated from periodontitis group (28%) than from the control group (13%) ($p$= 0.013), as previously reported [112]. CONS distribution was similar between both groups in Ireland [113]. The inflammation in periodontitis increases extracellular matrix components synthesis, facilitating adhesion and prolifiration of CoNS. While *S. aureus* can form biofilms, it struggles to survive long-term in the oral cavity compared to CoNS. CoNS exhibits greater antibiotic resistance than *S. aureus* [114], which enables them to persist in the oral environment.

Caries were more prevalent in females than males, as observed in Ethiopia [115], Canada [35], Germany [116], Mexico [117], and Turkey [118]. This is attributable to hormonal fluctuations during puberty, pregnancy, and menopause, affecting saliva flow [35], the earlier teeth eruption in girls [35], and genetics like variations in the X-linked amelogenin gene, which can disrupt dental enamel surface [119]. No marked sex differences were found in Hungary [120].

A greater proportion of caries patients exhibited suboptimal oral hygiene, which increases the caries likelihood [121].

Sugar consumption represents a risk factor for caries. Fermentable carbohydrates facilitate the cariogenic bacteria growth [122]. A total of 71% of caries patients reported consuming sugar (36% females, 35% males).

In caries group, 25% of strains were identified as *C. albicans*, which was not significantly different from the control group (21%) ($p$= 0.778), consistent with studies in Canada [123] and Germany [124]. *Candida* spp. distribution

differed between these groups in Turkey [125] and China [126]. *Candida albicans* synergizes with bacteria, creating an acidic microenvironment that facilitates enamel demineralisation [127]. In persons with robust immunity and in the absence of caries-associated bacteria, *C. albicans* is effectively controlled, and its amounts do not differ considerably between both groups.

*Staphylococcus aureus* was the most isolated bacterium in subjects with and without caries, with 18% and 24%, respectively ($p$= 0.336), consistent with results in India [128]. Its prevalence in Nigerian individuals with caries was 53% [129].

There were no significant differences in the *Enterobacteriaceae* isolation between healthy (11%) and patients groups (19%-3%) ($p$> 0.05), as observed in Colombia [130].

*Acinetobacter* spp. isolation was low (3%-2%) in all study groups, while *Pseudomonas* spp. were only isolated in the periodontitis and caries groups, with one strain from each (2%), as found in Japan [131-133]. These bacteria produce exopolysaccharides protecting them from host defenses [134].

*T. asahii* and *S. cerevisiae* accounted for 7% of the overall frequency. Their isolation rate was 1.23% in Iran [19], 5.6% for *T. asahii* and 19.4% for *S. cerevisiae* in Taiwan [135]. *Trichosporon* is an opportunistic pathogen causing superficial and invasive deep-seated infections [136].

In this study, 25 of the 74 subjects with oral diseases exhibited systemic diseases. A bidirectional relationship exists between systemic diseases and oral health [137]. Poor glycemia control in diabetes worsens periodontal diseases [138], while chronic inflammation in periodontitis may influence glucose metabolism and insulin resistance, contributing to the DM pathogenesis [139]. Thyroid disorders cause salivary glands inflammation, increase susceptibility to caries and periodontal disease [140], additionally periodontitis impact thyroid function [141]. Periodontal disease is a risk factor for cardiovascular disease, as it triggers inflammatory and immune responses, damaging epithelial tissue and affecting endothelial cells. Hypertension can lead to ischemia in the periodontium, worsening periodontal diseases [142], while altered salivary pH and viscosity in hypertensive patients can further impact oral health [143].

## Conclusions

Staphylococci and *Candida* were the most abundant microbes on the tongue's surfaces. The microorganisms distribution differed between individuals with and without oral diseases for certain species, while it was similar for others. Females exhibited greater susceptibility to caries, xerostomia, and gingivitis, whereas periodontitis was more detected in males.

For managing oral health, this study recommends improved oral hygiene, a balanced diet, probiotic consumption, and regular dental visits. Future research should include a larger and more diverse sample from various regions of Algeria to gain a more comprehensive understanding of the national oral microbiome.

## Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Patient consent statement

All participants, or their designated representative, provided informed consent.

## Authors' contributions

H. L. Conceptualization, investigation , formal analysis and writing – original draft, A.S. Conceptualization and supervision, Z. B.O. Methodology, A.B. Investigation. All authors read and approved the final version of the manuscript.

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