Original Article

Effect of probiotic bacteria on oral *Candida* in head- and neck-radiotherapy patients: A randomized clinical trial

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ABSTRACT

Objective: The aim of the study is to assess the effect of probiotic bacteria on oral *Candida* counts in cancer patients who are undergoing head- and neck-radiotherapy in a tertiary care center.

Study Design: The study was a randomized clinical trial including 90 patients who just completed head- and neck-radiotherapy.

Materials and Methods: Participants were randomly allocated into three equal sized groups, i.e., probiotics group, candid group, and combination groups. Oral rinse samples of the patients were collected before and after the intervention for the identification of *Candida*. The samples were incubated on Sabouraud's Dextrose Agar with Chloramphenicol at 37°C for 48 h, to assess the counts of colony-forming units/milliliter (CFU/ml) of *Candida* in saliva, and further on chrome agar plates to identify the *Candida* spp. Data were analyzed using mixed ANOVA to compare mean CFU/ml of *Candida* among three groups before and after the intervention.

Results: A total of 86 patients were included in the final analysis and there was a statistically significant reduction in mean *Candida* spp. Counts (CFU/ml) after intervention in all the three groups ($P = 0.000$) and significant reductions identified in both probiotic and combination therapy groups. Apart from reduction in *Candida albicans*, significant decrease in *Candida glabrata* and *Candida tropicalis* was observed after probiotics usage compared to other groups.

Conclusions: The present study suggests that probiotic bacteria were effective in reducing oral *Candida* spp which can be recommended alone or in combination with traditional antifungal agents for effective reduction in oral *Candida* in head- and neck-radiotherapy patients.

KEY WORDS: Antifungal, head- and neck-cancer, oral candidiasis

INTRODUCTION

The term "head- and neck-cancer" comprises a vast number of tumors with different histopathological characteristics arising from various anatomical sites such as the lip, oral mucosa, paranasal sinuses, salivary glands, pharynx, larynx, cervical portion of the esophagus, thyroid, parathyroid, and skin.[1] Over 200,000 new head- and neck-cancers are reported in India annually, and they account for 30% of all cancers in India and 75% of them had tobacco and its products as their etiological factors.[2] Majority of the head- and-neck cancer cases require radiotherapy as a primary treatment, as an adjunct to surgery, in combination with chemotherapy.[2]

Most of the patients with head- and neck-cancers receive a dose between 50 and 70 Gy with a therapeutic intent. This total dose is usually given as 2 Gy per fraction, once a day, 5 days a week, over a 5–7-week period.[3] Radiotherapy is performed in fractions knowing the fact that there is a difference in the responses of tumor tissue and normal tissue. In addition to antitumor effects, ionizing irradiation induces damage in normal tissues situated in the field of radiation. This becomes particularly evident in the head- and neck-region, a complex area composed of variety of tissues that respond differently to radiotherapy and being situated in this region, salivary glands are also susceptible to radiotherapy.[3,4]

Salivary glands should be comparatively radioresistant due to their slow cell turnover rate,

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but the changes in salivary flow rate and quality of saliva that occur shortly after radiotherapy indicate that the gland tissue is an acutely responding tissue.[5] As the radiotherapy continues, the degeneration of the acinar epithelium escalates and leads to the development of interstitial fibrosis. Serous acinar cells appear to be more sensitive to irradiation than mucous acinar cells and ductal cells.[6-8]

The most notable salivary changes are a decreased pH and buffering capacity, salivary electrolyte imbalance, and altered nonimmune and immune antibacterial systems.[9] The decreased production of immunoprotein and lysozyme levels are relatively more than the reduction in salivary flow rate and pH which results in a significant immunoprotein deficit.[10] Compromise in immunologic mechanisms and oral clearance will lead to poor host protection which in turn is substantially related to changes in the oral microbiota of irradiated patients.[10] Of the common radiation-induced changes in the oral microbiome, the most clinically significant changes are the increase of *Streptococcus mutans, Lactobacillus* species, and *Candida* species.[10] However, the most common microbial infection in the oral cavity during or shortly after radiotherapy is candidiasis.[11] Ramirez-Amador *et al.* reported that the prevalence of positive *Candida* cultures increased from 43% at baseline to 62% at completion of radiotherapy and to 75% during the follow-up period.[12]

Candida albicans is the predominant species in radiation-induced hyposalivation individuals. Transformation from a state of commensalism to that of pathogen by this organism is attributed to local and systemic factors. Hyposalivation may lead to a shift in the oral microbiota from symbiosis to dysbiosis, which may provide the opportunity for *Candida species* to multiply and cause infection in the oral mucosa.[13] Various oral antifungal agents such as clotrimazole, fluconazole, and ketoconazole. had been used to treat oral candidiasis.[14] However, effective they are in reducing oral *Candida* but they also cause adverse effects such as nausea, loss of appetite, and diarrhea.[15]

Probiotics are defined as microorganisms that promote benefits to host health, mainly by regulating resident microbiota. Probiotics have been consumed for many years for therapeutic and prophylactic reasons.[16] Probiotics had a potential to bring back equilibrium in an altered bacterial ecology in the human body.[16] All these years, probiotics had been used safely to treat antibiotic-induced diarrhea and vaginal candidiasis.[17] In a study conducted in elderly population, there was a decrease in the prevalence of *Candida* in oral cavity after the consumption of cheese enriched with probiotic bacteria.[18] Ishikawa *et al*. reported that probiotics were effective in reducing oral *Candida* colonization in denture wearers.[19] In another study conducted by Li *et al*. confirmed that inclusion of locally administered probiotics helped in reduction of *Candida* spp. in *Candida*-associated stomatitis.[20] The use of probiotics along with routine periodontal procedures was found to be an additional benefit in treating halitosis.[21]

Candidiasis being the most common disease among the patients who underwent head- and neck-radiotherapy, and considering adverse effects of conventional antifungal agents and the resistance of *Candida* spp. toward the azoles; the search for products that could help in its treatment is important. Hence, the hypothesis tested was "Probiotics has effect on oral *Candida* spp. (colony-forming units/milliliter [CFU/ml]) among head- and neck-radiotherapy patients."

MATERIALS AND METHODS

Design

A randomized clinical trial was conducted from May 2014 to September 2015.

Participants

Patients who underwent head- and neck-radiotherapy at City Cancer Centre, Vijayawada, Andhra Pradesh, India, were selected based on inclusion and exclusion criteria. A total of 114 patients were examined by an investigator (RD) for eligibility. Nearly, 90 patients fulfilled the inclusion criteria. Participants were randomly allocated into three equal sized groups as probiotic group, candid group, and combination group [Figure 1].

Inclusion criteria

- Patients who just completed (after 2 weeks of final dose) head- and neck-radiotherapy were included
- Patients who had not used antibiotics and antifungals for the previous 3 months
- Patients who gave written, voluntary consent to participate in this study.

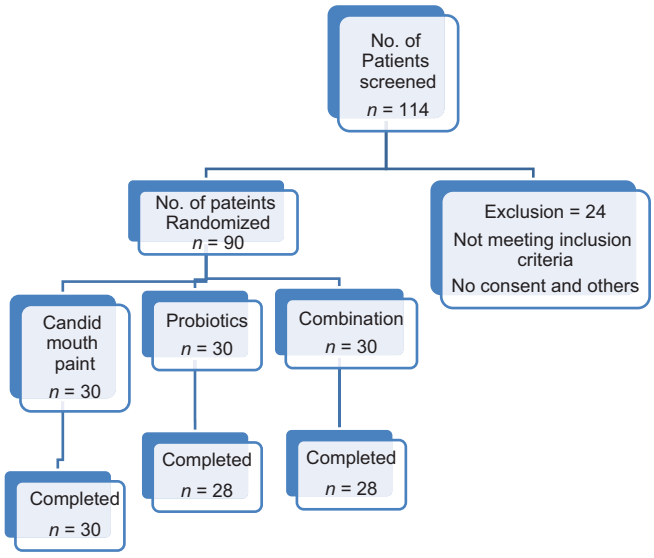

Figure 1: Flow chart of enrolment, randomization, and intervention and follow-up of the participants

Exclusion criteria

- Patients with emergency conditions/critically ill were not included and have been treated immediately. If any new fact that could influence the results (e.g., antibiotics use) emerged during the study, the samples of that patient were also excluded.

Sample size

Based on the fact that changes in mean CFU/ml in a study conducted by Mendonça *et al.*,[22] the sample size was calculated and considering 95% CI and 80% power, 80 individuals were sufficient to detect clinically significant difference of 10%. The final sample size was adjusted to 90, expecting the fact that there are 10% chances of attrition.

Randomization and blinding

Randomization and blinding were performed with the help of sealed, opaque, individually numbered envelopes, restricted to choosing one at a time. The envelopes contain information regarding the group allocation and the identification number. Patients were asked to pick any concealed envelope randomly from the envelope pool. Allocation concealment was independently maintained by a Research Committee Member of the Drs. Sudha and Nageswara Rao Siddhartha Institute of Dental Sciences, India. Patients, outcome assessors (microbiologist), and the data analysis were blinded for the trial intervention. The microbiologist was therefore neither part of the investigative team that performs the trial intervention, nor took part in the salivary sample collection of the patient and had no access to the randomization sheets. All salivary samples were collected by a single investigator (SV) who was not aware of the group allocation of the patients. Blinding of patients was not feasible, as the probiotic sachet is different from candid mouth paint and combination system.

Intervention

All participants were fully informed about the objectives and methodology of the study. Initial salivary samples were collected from all the participants. Test group participants were given probiotic sachets, Darolac® manufactured by Aristo Pharmaceuticals Pvt. Ltd., India. A single sachet contained at least 1.25 billion live cells of a blend of four probiotic strains: *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium longum*, and *Saccharomyces boulardii*. Participants were instructed to mix the powder with liquid three times daily, at the same hour, for 30 days. Participants were asked to swish for 2–3 min and swallow the above solution. Antifungal agent, clotrimazole 1% (candid mouth paint) was advised for the second group for prescribed number of days as per regimen, and for third group, both probiotics and candid were given. After this period, another saliva sample was collected from each participant.

Risks

Apart from adverse effects of antifungal agents, no additional risks for study patients are anticipated, since the safety and feasibility of use of probiotics in humans have been established in several previous studies.[19,20,23] Probiotics used in this trial were already certified and have been in market from several years.

Outcome measures

The primary efficacy endpoint measure of this trial is the difference in salivary *Candia spp* count (CFU/ml) between pre- and post-intervention and difference in prevalence of different species of *Candida* was the secondary endpoint measure.

Data management

All relevant data collected during the trial were entered into a case record sheet by the investigator as soon as information was collected. The investigator (RD) was responsible for the accuracy of the documentation and the confidentiality was maintained. An explanation was written on case sheet for all missing data.

Safety evaluation and reporting of adverse events

An adverse event is defined as any untoward medical occurrence in a patient that does not necessarily have a causal relationship with the trial treatment and that occurs between inclusion of the patient and final visit. No adverse effects were reported in this study.

Withdrawals

Patients had a choice to withdraw from this clinical trial on their own request at any time and without giving reasons for their decisions. Withdrawals were documented in the case record form and reasons (if any) were also mentioned.

Isolation, counting, and identification of *Candida*

Before starting the study, 2 ml of sterile saline was used to rinse the mouth for 30 s and they are advised to spit into a sterile disposable container for the semi-quantitative determination of the *Candida* counts.[24] Saliva samples were collected using oral rinse method by a SV. Then, a fraction (inoculating loop used in microbiology laboratory) of the samples was plated in culture media for isolation and identification of *Candida* yeasts. The samples were plated in Sabouraud's Dextrose Agar with Chloramphenicol (1 mg/ml) and incubated at 37°C for 48 h. The number of CFU/ml of saliva was counted using the formula: CFU/ml = 1000 × no. of colonies/4[25] Suggestive colonies from each plate were confirmed by a wet mount preparation using gram stain with glass slide and cover slip and was observed under microscope. The presence of budding and pseudohyphae formation of yeast cell is clearly differentiated in Gram-stained smear.[24,25] The *Candida* species were identified by Hi Chrome *Candida* differential agar (M-1297A).

Trial organization and administration

Ethical approval

Before the start of the trial, the study protocol was approved by Institutional Review Board of Drs. Sudha and Nageswara Rao

Siddhartha Institute of Dental Sciences, and all the procedures followed were in accordance with Helsinki Declaration.

Registration

Protocol of this trial has been registered at Clinical Trials Registry-India, Reference number: CTRI/2018/02/011812.

Good clinical practice

All the procedures mentioned in this trial protocol, relating to the conduct, outcome assessment, and documentation of this trial are designed to ensure that the trial abide by the Indian Council of Medical Research and the ethical principles set by the Declaration of Helsinki.

Statistical analysis

Data were analyzed with Statistical Software for Social Sciences, SPSS for windows. version 20.0 (Armonk, NY: IBM corp). First, the normality of the data (CFU/ml counts, pre- and post-intervention of probiotics) were evaluated using the K-S test and data were found to be normally distributed. Chi-square test was used for bivariate categorical variables. Mixed ANOVA and Tukey's HSD *post hoc* test were used for multiple comparisons. $P \leq 0.05$ was considered as statistically significant for all the analysis.

RESULTS

A total of 114 patients were screened, out of which 90 patients who met inclusion criteria were included in this study. Selected patients were randomly divided into three groups: −30 in candid group +30 in probiotic group +30 in combination of probiotics and candid. Finally, data were collected from 86 patients and four patients were excluded from the study due to protocol deviation (lack of compliance) and loss of follow-up [Figure 1].

Demographic profile of the study participants

The final analysis included a total of 86 patients, among which 58 were male and 28 were female. This study comprised of participants of age group between 24 and 80 years. The mean age of the participants is 53 ± 13.5 years [Table 1].

Comparison between mean Candidal count (colony-forming units/milliliter) between various groups

The mean baseline Candidal (CFU/ml) value was 4883.3 ± 1731 for candid group, 5339.2 ± 1194 for probiotic group, and 5687 ± 1397 for combination group. The mean postintervention candidal (CFU/ml) values were 670 ± 52 for candid group, 400 ± 36 for probiotic group, and 230 ± 42 for combination group, respectively [Table 2 and Figure 2].

There is a reduction in mean candidal count (CFU/ml) in all the three groups between baseline and postintervention analysis which was statistically significant (mixed ANOVA, $P = 0.000$) [Table 3], and Tukey's *post hoc* analysis indicates that significant reduction occurred in both probiotic and combination groups compared to candid group, but no statistically significant difference between these two groups was present ($P = 0.330$) [Table 4].

Table 1: Age-gender distribution of the study participants

Age group (years)	Gender		Total (%)
	Male	Female	
20-30	6	0	6 (7)
31-40	9	3	12 (14)
41-50	14	4	18 (21)
51-60	11	14	25 (29.5)
61-70	14	4	18 (21)
71-80	4	3	7 (7.5)
Total (%)	58 (67.4)	28 (32.6)	86 (100)

Mean age of the participants is 53±13.5 years (range: 24-80)

Table 2: Comparison between mean *Candida* count (colony-forming units/millilitre) between various groups

Group	*n*	Preintervention	Postintervention
Antifungal (candid)	30	4883.3±1731	670±52
Probiotic	28	5339.2±1194	400±36
Combination (both probiotics and antifungal agent)	28	5687±1397	230±42

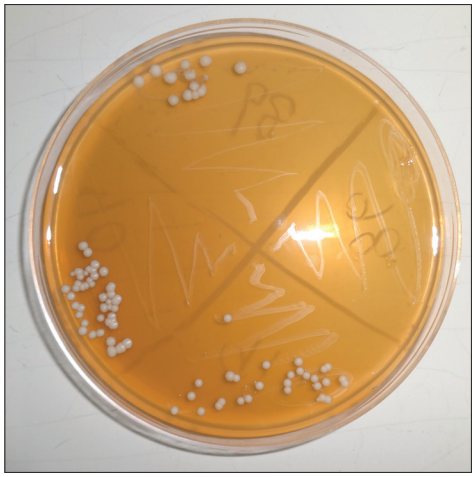

Figure 2: *Candida* colony-forming units

Comparison of *Candida* species at base line and after intervention among study groups

The predominant species among all the three groups was *C. albicans* which was isolated from all the samples. Overall, postintervention reduction in *C. albicans* was observed in all the three groups quantitatively [Figure 3].

There were seven samples in probiotic group with mixed species, out of which *Candida tropicalis* and *Candida glabrata* were present in all seven samples, *Candida parapsilosis* in three samples and two more samples have *Candida krusei*. Apart from reduction in *C. albicans*, significant decrease in *C. glabrata* (7–3) and *C. tropicalis* (7–4) was observed after probiotics usage [Figure 4].

In candid mouth paint group, six out of 44 isolates had mixed species, and in post intervention samples, apart from *C. albicans*,

significant reductions were observed only for *C. tropicalis* and other species were less affected with intervention.

Six out of 45 isolates were mixed species in combination group and again significant reductions were observed for *C. tropicalis*. *C. glabrata* was less affected with intervention [Table 5].

DISCUSSION

Increase in head- and neck-cancer prevalence is an alarming health problem in India, and radiotherapy plays a major role in treating such cases. Improving quality of life of treated patients is becoming more and more important.[26] It was not unusual to identify a high prevalence of the *Candida* species in the oral cavity of these patients.[27]

Salivary IgA antibodies inhibit the adherence of candidal cells to oral epithelial cells. However, there is a critical concentration of fungi above which salivary antibodies are ineffective.[28] The anti-candidal activity of the microbiome is an acquired host defense mechanism that plays a very important first line of defense role in protecting humans against candidiasis since the microbiota in the intestine not only can inhibit the growth of

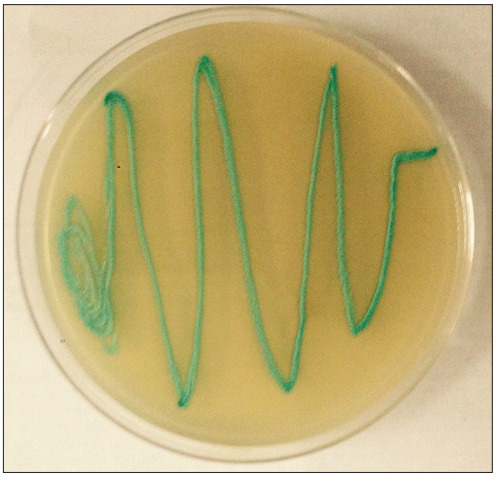

Figure 3: *Candida albicans* on Hi Chrome *Candida* differential agar

Figure 4: Various species of *Candida* on Hi Chrome *Candida* differential agar

Table 3: Comparison between group means with mixed ANOVA

Source	Tests of within-subjects contrasts				*F*	Significant
	Time	Type III sum of squares	df	Mean square		
Time	Linear	1,018,715,454.816	1	1,018,715,454.816	1264.208	0.000
Time × group	Linear	11,351,557.863	2	5,675,778.931	7.044	0.001
Error (time)	Linear	66,882,511.905	83	805,813.396		

Table 4: *Post hoc*: Tukey's honestly significant difference

(I) group	(J) group	Mean difference (I-J)	SE	Significant	95% CI	
					Lower bound	Upper bound
Candid	Probiotics	270.00000	116.58893	0.059*	−8.2365	548.2365
	Combination	439.64286*	116.58893	0.001*	161.4063	717.8794
Probiotics	Candid	−270.00000	116.58893	0.059*	548.2365	8.2365
	Combination	169.64286	118.58204	0.330	113.3502	452.6359
Combination	Probiotics	−439.64286*	116.58893	0.001*	717.8794	161.4063
	Candid	−169.64286	118.58204	0.330	452.6359	113.3502

SE=Standard error, CI=Confidence interval

Table 5: Comparison of *Candida* species at pre- and post-intervention among study groups

Species	Probiotic group (*n*=30), *n* (%)		Candid group (*n*=28), *n* (%)		Combination (*n*=28), *n* (%)	
	Before	After	Before	After	Before	After
Candida albicans	30 (61)	26 (79)	28 (64)	27 (71)	28 (62)	25 (73)
Candida tropicalis	7 (14)	4 (12)	5 (11)	3 (8)	6 (13)	2 (7)
Candida parapsilosis	3 (6)	0	4 (9)	3 (8)	5 (11)	3 (9)
Candida glabrata	7 (14)	3 (9)	6 (14)	5 (13)	4 (8)	3 (9)
Candida krusei	2 (4)	0	1 (2)	0	2 (4)	1 (2)
Total	49 (100)	33 (100)	44 (100)	38 (100)	45 (100)	34 (100)

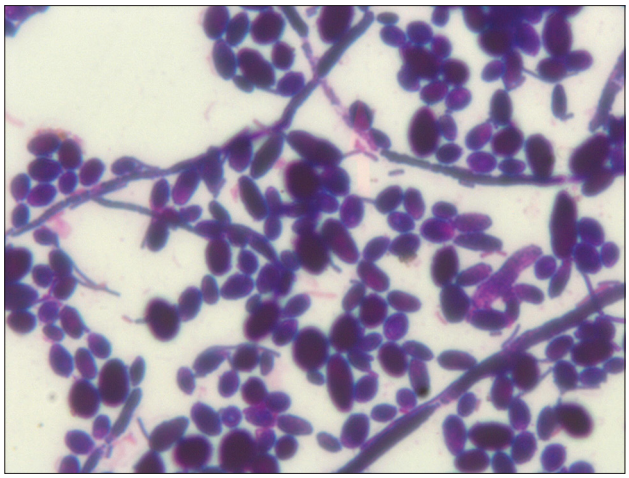

Figure 5: Gram-stained smear showing budding in yeast cells

Candida species, but they can also interfere with the capacity of the pathogenic yeast to adhere to epithelial tissues.[28]

Microbial cell-to-cell communication plays an important role in the colonization process. The multicellular lifestyle of bacterial and yeast biofilms is induced by environmental stress and/or restricted supply of nutrients. This cooperation leads to adaptation to natural stress and the involved bacteria and yeasts interact through quorum sensing (QS).[29] QS is polymicrobial coordination within a microbial community, based on excreted small molecules triggering a genetic response when present in sufficiently high concentrations. QS occurs both in single species bacterial communities and in complex mixed bacterial-yeast communities. A recent study showed that *Candida* hyphal formation can be inhibited by Gram-negative bacterial QS molecules.[29] Elahi *et al*. conducted a study to correlate between interleukin-4 secretion and *C. albicans*, and the results of the study showed that enhanced interleukin-4 was responsible for reduction in *Candida* count.[30]

Probiotic microorganisms such as some *Lactobacillus* species have been described as promoting a healthy digestive system and immunomodulation.[31] There is reasonable evidence that some probiotic species may also have a beneficial effect in treating gastrointestinal disorders and vaginal candidiasis.[31,32]

Probiotics had been used in oral health and diseases, and studies reported that they were effective in reducing oral *Candida* colonization in denture wearers and reduction of *Candida* spp. in *Candida*-associated stomatitis.[19,20,33,34] The use of probiotics along with routine periodontal procedures was found to be effective in treating halitosis.[21] For example, a study conducted by Stensson *et al*., in which children were prescribed probiotics during 1st year of life and there is significant decrease in dental caries in primary dentition evaluated at the age of 9 years.[35]

The literature that is available on the effect of probiotic consumption on oral *Candida* among head- and neck-radiotherapy patients was found to be limited. Hence, a randomized, double-blinded clinical trial was conducted to assess the effect of probiotics on oral *Candida*. In this study, one test group (probiotic) was compared with antifungal agent (candid) and combination (probiotics + candid) groups, and no placebo is used because of ethical considerations. Of 90 participants, 86 patients were available for the final assessment and reasons for dropouts have been already mentioned in the flow chart [Figure 1], however, none of the dropouts are due to the adverse effects of interventions.

Among the study participants, majority of them (67.4%) were male and 32.6% were female [Table 1]. It is not unusual to find more number of head- and neck-cancer cases in males because of tobacco consumption habits, and similar findings were reported by Dahiya *et al*.[36]

The postintervention results showed a reduction in the mean Candidal CFU/ml in all the three groups, especially, in probiotic group and combination group. Similar results were observed in a study conducted by Mendonça *et al*.,[22] where probiotic Yakult LBz (®) (*Lactobacillus casei* and *Bifidobacterium breve*) was used in 42 healthy adults, and the study conducted by Dos Santos *et al*. in younger population.[37] Hasslöf *et al*.[38] also studied the effect of commercially available probiotic bacteria on oral *Candida* and *S. mutans*.

Results suggested that there are a decrease in prevalence of *Candida* after probiotic consumption. In this study, no placebo group is included due to ethical concerns of denying treatment for patients who were positive on culture with oral *Candida* [Figure 5]. However, the primary endpoint measure (salivary *Candida* count) cannot be influenced by the subjective assessment of the patient. Person who involved in collection of salivary samples and microbiologist who cultured and counted *Candida* CFU were blinded. In the entire study, uniformity was maintained regarding the time for collection of the salivary samples in every patient and the time for processing, thus decreasing the risk of interferences.

Among the species identified in this study, *C. albicans* was the most prevalent species before and after interventions. Its predominance among the other species of *Candida* in humans had been demonstrated. Several nonalbicans species were also isolated, a phenomenon commonly observed in immunocompromised, chronic pathologically ill patients and who were under prolonged course of antibiotics.[38,39] For example, Shrestha *et al*.[12] conducted a study in patients who are undergoing head- and neck-radiotherapy, they observed that apart from *C. albicans*, several nonalbicans species such as *C. krusei*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* were observed with a frequency of 10%, 6.66%, 3.33%, and 3.33%, respectively. Dahiya *et al*.[36] observed that 30% of the candidal infection was caused by nonalbicans species in head- and neck-cancer patients receiving external beam radiotherapy.

In the present study, 7 of 49 isolates in probiotic group, 6 of 44 in candid group, and 6 of 45 isolates in combination group were mixed species at baseline. After intervention, along with reduction in *C. albicans*, nonalbicans species also decreased, especially, in probiotic and combination group. Among the nonalbicans species, significant reductions were observed for *C. glabrata*. These findings were similar to study conducted by Hatakka *et al*.[18] and Dos Santos *et al*.[37]

The isolation of nonalbicans species in head- and neck-radiotherapy patients is a cause for concern because most of these species were resistant to conventional antifungal agents and are able to cause topical as well as systemic infection. However, in this study, it is noteworthy to mention that there was significant reduction in nonalbicans species in both probiotic and combination groups, especially *C. glabrata* at the end of the interventions.

In the present study, considerable rates of reduction in the number of isolates of *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* as well as other yeasts species, were verified at the end of the treatments. This suggests that probiotics were effective in reducing more complex species. Probiotics can improve the defense function of oral epithelial cells through cytokine induction.[40] Regaining and maintenance of the oral defense system and microbial ecology, frequently compromised in head- and neck-radiotherapy patients, is the best alternative to prevent the candidiasis and other opportunistic diseases.

The principal advantage of probiotics is that, unlike antifungal agents, development of resistance to probiotics is less likely due to its mechanism of action.[40] Another significant advantage is lack of potential toxic or adverse effects of probiotics with added utility in preventing chemotherapy-induced Gastrointestinal tract (GIT) disturbances.

An increased level of *Candida* species is generally associated with microbial dysbiosis and salivary dysfunction. Probiotic approaches in preventing oral candidiasis aim to alter dysbiosis, and restore a symbiotic ecosystem between the bacterial community and the fungal community in the oral cavity. The results in this and similar studies indicate that probiotics can reduce the prevalence of oral *Candida*. However, it is yet not recommended for the treatment of oral candidiasis. Being a carrier of *Candida*, as part of the normal microbiome, is quite normal and in most cases does not lead to oral candidiasis. However, a reduction in the amount (prevalence) of oral *Candida* species by probiotic bacteria may help preventing the *Candida* species from being infectious by reducing their number.

The findings of this study suggest that the consumption of probiotics had beneficial effects in reducing the pathogenic oral *Candida* species with less/no adverse effects in head- and neck-radiotherapy patients.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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