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CAN PROBIOTICS BE A POTENTIAL GAME-CHANGER IN REDUCING MUCOSAL TOXICITY DURING HEAD AND NECK RADIOTHERAPY?

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Abstract – *Objective:* Radiation-induced mucositis (RIM) is an important cause of unplanned treatment breaks during definitive chemo-radiotherapy (CTRT) in locally advanced head-neck cancers (LAHNC). The accelerated repopulation that can occur during this period has proven to significantly compromise local control. Probiotics are currently being evaluated as immunomodulators that, in addition to anti-inflammatory effects, maintain the integrity of mucosal barriers. The current study intends to identify the possible mechanisms by which probiotics can reduce the most important treatment compromising toxicity of radical CTRT, that is, RIM. It is also intended to explore the additional immune enhancement that probiotics can provide by quantifying CD4 T and CD8 T during the course of treatment.

Patients and Methods: Patients with LAHNC were randomized to the probiotic arm (A) or control arm (B). A radical dose of 70 Gy equivalent was planned with concurrent platinum-based chemotherapy. All patients underwent CD4 T and CD8 T cell counting pre- and post-completion of treatment. RIM was documented and analyzed in both arms.

Results: 31 patients were enrolled; arm A (n=9/15) and arm B (n=15/16) were available for analysis. Arm A showed a 57% relative reduction in grade 3 mucositis compared to Arm B. The mean weight loss in Arm A was 4.6 ± 3.08 kg *vs*. Arm B was 7 ± 2.3 kg ($p = 0.05$). A complete response was noted in Arm A at 77% (7/9) *vs*. Arm B at 33% (5/15). A fall in the CD4 T cell proportion of A = 9% (p=0.08) and B = 5% (p=0.05) was noted post-CTRT. CD8 T cell proportion rose by A = 13% (p=0.04) and B = 10% (p=0.001).

Conclusions: Probiotics reduce RIM in LAHNC and enhance the antitumor CD8 T cells, thus suggesting a window for an affordable, non-toxic immunomodulator.

Keywords: Probiotics, Mucositis, Head and Neck Cancer, Radiotherapy.

INTRODUCTION

Definitive concurrent chemo-radiotherapy (CTRT) is the standard of care for patients with locally advanced head and neck cancers (LAHNC)[1]. The probability of attaining optimal local control is directly related to the timely completion of the planned radiation protocol. Unplanned treatment breaks are associated with the risk of accelerated repopulation and dilution of treatment efficacy both in terms of primary response and survival[2]. Radiation-induced oro-pharyngeal mucositis is an important cause of such treatment breaks and critical weight loss, especially during the last few weeks of head-neck radiotherapy[3].

Emerging experimental and clinical data suggest that probiotics have the potential to significantly reduce the incidence as well as intensity of oral mucositis[4]. They are safe, well tolerated, cost-effective, and devoid of significant serious side effects[5].

Immunotherapy in the current era has several established agents directed at transforming cold tumors into hot tumors through increased lymphocytic infiltration[6]. However, they come with a range of toxicities, some of which can significantly impact patients' quality of life. The other caveat is the requirement of a favorable tumor profile to be suitable for immunotherapy agents. Many immunotherapeutic agents are not easily available to patients in Low and Middle-income countries (LMIC) where head and neck malignancies have a higher incidence. Currently, the cost to each individual patient far exceeds the cost of traditional modalities.

Probiotics, on the other hand, are rapidly gaining importance as a therapeutic approach that has the potential to solve several of these issues[7,8]. Research has also proven their capacity to potentiate dentritic cells and enhance T cell activity[9]. Some rationales for using probiotics in ameliorating mucositis include their anti-inflammatory action, competitive elimination of pathogenic bacteria, and maintaining the integrity of the mucosal barrier. Few studies[10,11] have also reported pro-apoptotic effects. However, there is a paucity of randomized controlled trials to establish the efficacy of probiotics in the scenario of either reduction of mucositis or potentiating anti-tumor immune response. Most of these studies have addressed the role of probiotics in hematological malignancies. Very few studies have addressed the role of probiotics in head and neck cancers[4,12,13].

The current study intends to address the impact of probiotics on radiation-induced mucositis (RIM) for patients receiving concurrent chemo-radiotherapy in LAHNC.

PATIENTS AND METHODS

Patients referred to the Department of Radiation Oncology for the treatment of locally advanced head-neck cancers were selected. The study was approved by the ethical committee Kidwai Cancer Institute Medical Ethics Committee of the Kidwai Memorial Institute of Oncology] with protocol number [KCI/MEC/019/14.November.2019 and date 22/11/2019. Patients in both cohorts provided written informed consent according to the principles of the Declaration of Helsinki. The patients were randomized using the random number table method into the drug arm (A) or the control arm (B).

Inclusion criteria: Patients with locally advanced head-neck cancers (oropharyngeal, hypopharyngeal, and laryngeal sites; stage III & stage IV A) who were eligible to receive concurrent chemoradiotherapy were included in this study. All the patients were between 18 and 70 years old with an Eastern Cooperative Oncology Group (ECOG) Performance status of 0-2.

Exclusion Criteria: Patients who have previously received radiation in the same area or who have second malignancies were excluded. Other exclusion criteria included patients with uncontrolled co-morbidities and those who were immunocompromised (HIV+).

Arm A (probiotic arm): patients received the probiotic formulation in three Sachet two times a day from one week before the start of concurrent chemoradiotherapy to the end of treatment. The probiotic used contained a combination of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium longum*, *Saccharomyces boulardii* to a total count of 2.75 billion CFU, and Fructooligosaccharides 100 mg.

Arm B (control): patients in the observational cohort did not receive the probiotic drug.

Peripheral Blood Lymphocyte Phenotype Detection: All patients underwent a baseline evaluation of immune parameters (CD4 T, CD8 T cells) by flow cytometry one week before the initiation of treatment and within one week after the end of treatment. Venous blood samples were drawn before initiation of CTRT and within one week of completion of CTRT in both arms. Four-color flow cytometric analysis of peripheral lymphocytes was performed. In this procedure, the subjects' sample was mixed with 20 µL of BD Multitest CD3/CD4/CD8/CD 45 reagent. All samples were mixed gently and then incubated for 15 min in the dark at room temperature. Post incubation, 450 µL of lysing solution was added to each tube. The samples were then washed with a staining buffer to remove unbound antibodies. These were then analyzed by flow cytometry.

CD45 is a ubiquitous cell surface marker of immune-regulating hematopoietic cells in the peripheral blood. T cells were defined by CD3 expression, and B cells by CD19 expression. T-lymphocyte subsets were identified by the presence of CD4 and CD8.

To provide a quality check on the consistency of analytics, we evaluated these same parameters in seven patients of carcinoma cervix receiving concurrent chemoradiation. These patients did not receive probiotics.

Patients in both arms received external beam radiotherapy to a dose of 70Gy/35Fr along with weekly concurrent cisplatin chemotherapy. External beam radiotherapy (EBRT) was delivered by intensity modulated radiotherapy (IMRT) using the simultaneous integrated boost (SIB) technique.

Patients in both arms were observed for the incidence of oral mucositis and graded using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) Version 5.0. Nutritional assessment was done in terms of weekly weight monitoring and documentation of any weight loss in both arms. Any interruption in treatment delivery as a direct correlate of mucositis was documented and compared in both arms.

Statistical Analysis

The results of continuous measurements were studied by mean ± standard deviation (SD). Student's *t*-test was used to evaluate the difference between the two groups for continuous variables. *p*-value less than 0.05 was considered statistically significant.

RESULTS

Thirty-one patients were enrolled, fifteen in experimental Arm A and sixteen in control arm B. Nine out of fifteen in arm A and fifteen out of sixteen in arm B were available for analysis. Two patients in the experimental arm died of non-cancer-related causes during treatment. Four patients in the experimental arm and one patient in the control arm discontinued treatment and follow-up for personal reasons.

The mean age of the recruited patients was 61 ± 4 years, of which 4/9 (44%) patients were stage III and 5/9 (56%) patients were stage IV in the study arm. In the control arm B, 7/15 (47%) were stage III, and 8/15 (53%) were stage IV. Patients in both arms, A & B, were able to receive 80% of the planned concurrent chemotherapy. The baseline comparison of Absolute lymphocyte counts was comparable between both arms (3865 ± 1166 cells/microlitre in Arm A *vs.* 4136.38 ± 1485 cells/microlitre in Arm B (p=0.5) (Table 1).

Mean weight loss in Arm A was 4.6 ± 3.08 kg *vs.* Arm B was 7 ± 2.3 kg (p = 0.05). Arm A showed a 57% relative reduction in grade 3 mucositis compared to Arm B. Pattern of mucositis is shown in Figure 1.

In arm A, a complete response (CR) was observed in 77% (7/9) of the patients, whereas in Arm B, this was inferior; only 33% (5/15) achieved a CR. The partial response noted in arm A was 22% (2/9). In arm B, the partial response was 33% (5/15). However, there was progression in 13% (2/15) of patients in arm B (2/15) (Figure 2).

Immuno-profiling post CTRT:

The CD4 & CD8 T cell proportion was calculated by the given formula:

- CD4 T Cell/ CD45 T cell
- CD8 T Cell/ CD 45 T cell, respectively.

TABLE 1. TRENDS OF ABSOLUTE LYMPHOCYTE COUNTS.

Parameter	CTRT + Probiotics (A)	CTRT (B)	Unpaired *t*-test
Pre-treatment (absolute) (cells/microlitre) mean ± SD	3865 ± 1166	4136 ± 1485	p = 0.05
Post-treatment (absolute) (cells/microlitre) mean ± SD	1238 ± 674	1369 ± 573	p = 0.09
Mean reduction in absolute lymphocyte counts (%)	54.33 ± 27.30	72.85 ± 13.49	

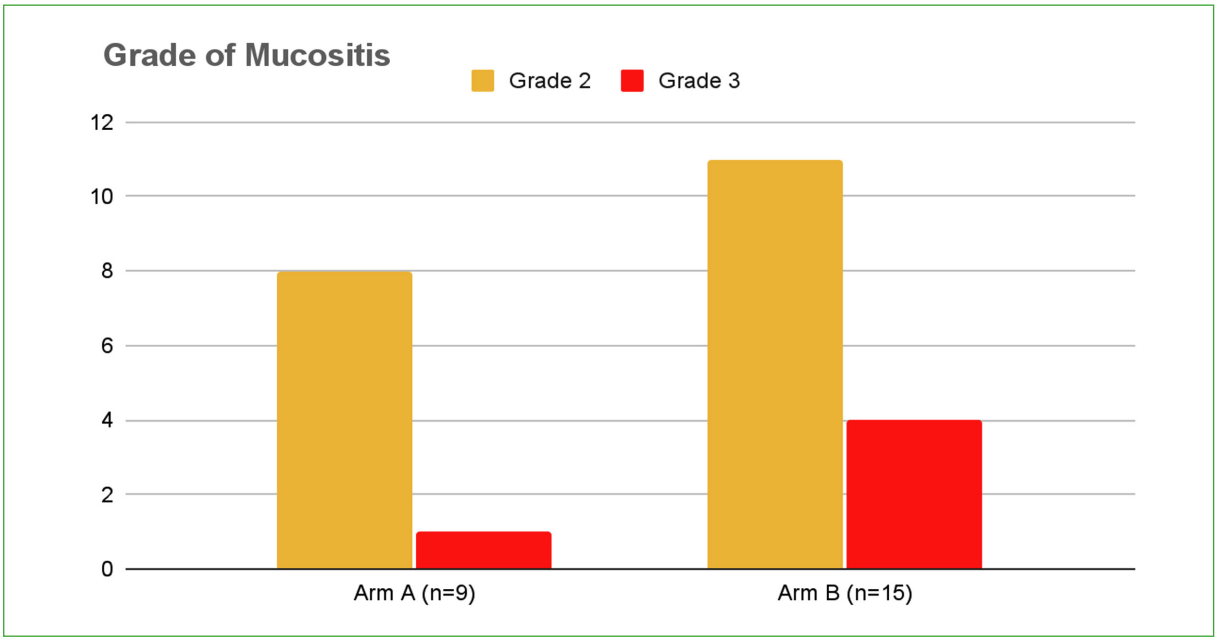

Figure 1. Comparison of mucositis between Arm A (CTRT + Probiotics) *vs.* Arm B (CTRT).

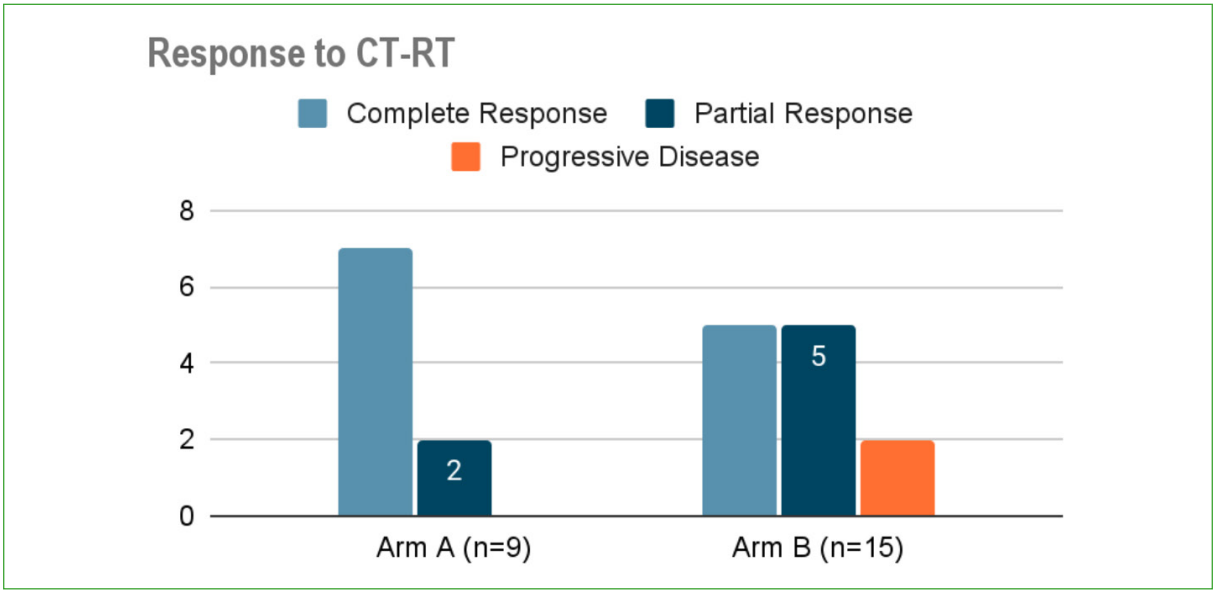

Figure 2. Comparison of Response to CT-RT between the two arms.

A uniform fall in the CD4 T cell proportion of 9% in Arm A (p=0.08) and 5% in arm B (p=0.05) was observed.

A uniform rise in the CD8 T cell proportion in arm A of 13% (p=0.04) *vs.* arm B of 10% (p=0.001) was noted, which could be interpreted as a surrogate marker of immune activity in the tumor microenvironment (Table 2 and 3; Figure 3A, 3B, 3C).

The patterns of change of CD4 and CD8 Tcell proportions post CTRT were also calculated for our observational cohort of cervical cancer patients. A similar pattern of fall in the CD4 T cell proportions and a significant rise in the CD8 T cell proportion post-CTRT were observed in these patients (Table 4). This confirmed that this is an anticipated trend and the comparatively larger fall of CD4 T cell proportions in both cohorts need not reflect a compromise on adaptive immunity.

TABLE 2. TREND OF CD4 T CELL PROPORTION.

Parameter	CTRT + Probiotics	CTRT
Pre-treatment mean ± SD	0.44 ± 0.0.08	0.38 ± 0.06
Post treatment mean ± SD	0.35 ± 0.12	0.33 ± 0.08
Trend	9% decrease	5% decrease
p value (paired *t* test)	0.08	0.05

TABLE 3. TREND OF CD8 T CELL PROPORTION.

Parameters	CTRT + Probiotics	CTRT
Pretreatment mean ± SD	0.28 ± 0.12	0.33 ± 0.07
Post treatment mean ± SD	0.41 ± 0.14	0.43 ± 0.09
Trend	13% rise	10% rise
p value (paired *t* test)	0.04	0.001

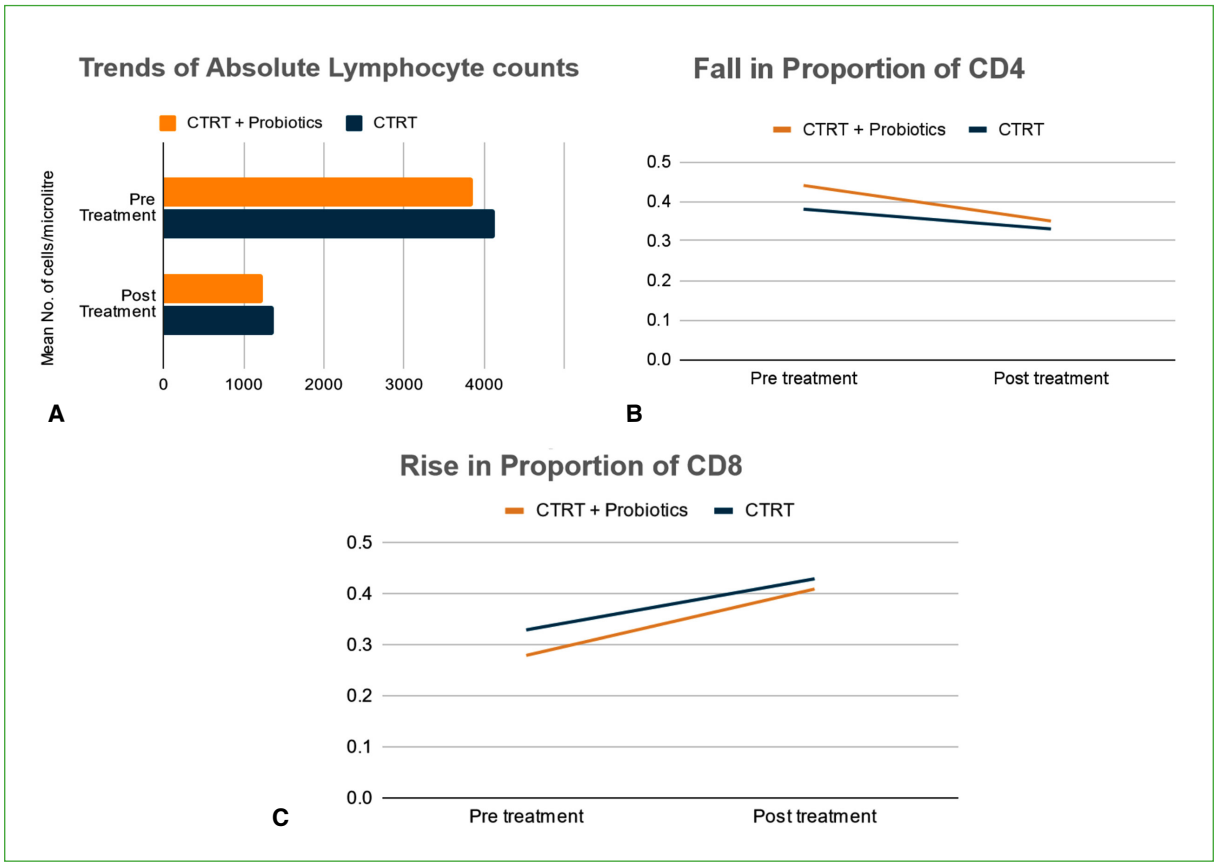

Figure 3. A, Trends of absolute lymphocyte counts in both arms. **B**, Trend of CD4 T cell proportion. **C**, Trend of CD8 T cell proportion.

TABLE 4. CD 4 AND CD 8 T CELL PROPORTION IN PATIENTS WITH CA. CERVIX UNDERGOING DEFINITIVE CT-RT.

Parameters	CD4 T cell proportion	CD8 T cell proportion
Pretreatment % mean ± SD	0.39 ± 0.06	0.29 ± 0.08
Post treatment % mean ± SD	0.30 ± 0.14	0.42 ± 0.13
Trend	9% decrease	13% rise
p-value	0.10	0.001

DISCUSSION

Concurrent chemoradiation in head and neck cancers is associated with a high degree of acute mucositis, which can negatively impact the quality of life. The incidence of acute mucositis in head and neck cancer treatment can range from 80%-90%, out of which nearly 60% have grade 3 mucositis[14,15]. This is the most important contributing factor towards treatment interruptions during radiotherapy. Such breaks can compromise patient outcomes in terms of local control and survival. Treatment breaks result in accelerated repopulation of tumor cells that dilutes the effect of radiotherapy to eliminate all significant clones. It also contributes to increased hospitalization and the need for parenteral nutrition, narcotic analgesics, and antibiotics, thus increasing healthcare costs[15]. Nonzee et al[16] have demonstrated an incremental cost of nearly $18,000 attributable to mucositis-related hospitalizations for supportive care. The pattern of financial implications remains similar in developing nations, although the same has not been formally documented.

Chemoradiation significantly contributes to weight loss, which is an established poor prognostic factor in Head and Neck cancer cure. Langius et al[3] studied the pattern of weight loss in 1,340 patients of LAHNC undergoing radiotherapy with chemotherapy. Critical weight loss was defined as a loss of >5% body weight during radiotherapy treatment. The 5-year overall survival for patients with critical weight loss during RT was 62% in comparison with 70% for patients without critical WL (p=0.01)[3]. In our study, mean weight loss was lesser in the experimental arm (4.6 ± 3.08 kg) *vs.* the standard CTRT control arm (7±2.3 kg) (p = 0.05), which could be attributed to the lower rates of oropharyngeal mucositis and protection provided by probiotics in arm A.

In healthy mucosa, the coordination of commensal microbiota with innate and adaptive immune responses contributes to the establishment of immune tolerance and maintenance of the normal epithelial barrier[17,18]. Tissue homeostasis, as a part of innate immunity, is maintained by the epithelial cells expressing receptors for microbial identification of pathogen-associated molecular patterns (PAMPs). Chemoradiotherapy can dysregulate the multilayered interaction between microbiota and innate immunity, resulting in mucositis[19].

Lymphocytes account for about 30% of the normal human white blood cell population and play an essential role in antitumor immunity. The CD4 T cells are a part of the adaptive immune response. They interact with the surrounding microbiota to distinguish between useful and harmful microbes[19]. An imbalance in the natural microbiome environment is called 'dysbiosis.' Chemo-irradiation contributes to this dysbiosis, resulting in an overgrowth of pathogenic microbial organisms. The overgrowth of *Actinobacillus*, *Mannheimia,* and *Streptobacillus,* have been associated with the severity of oropharyngeal mucositis[17-20]. A dysbiotic microbiome is implicated in disturbing the regulation of Th1/Th17/Treg and Th1/Th2 balance, leading to accentuated mucositis during anti-cancer treatment. Similarly, the dysbiotic microbiome causes amplified immune and inflammatory responses, impairing tissue repair/regeneration[21].

Thus, chemoradiotherapy-induced OM results from amplified inflammatory responses, impaired cell proliferation, and increased cell senescence/apoptotic cell death[15,22]. These complex pathophysiological processes could be divided into five stages. The initiation/primary stage: here, the damage response is induced by generating reactive oxygen species (ROS) in response to cytotoxic agents. The message generation stage involves NF-κB activation, which subsequently upregulates several inflammatory cytokines, such as tumor necrosis factor-α (TNF-α),

interleukin (IL)–6, and IL-1β, cyclooxygenase-2 (COX-2), inducible Nitric oxide NO-synthase, and superoxide dismutase. These inflammatory cytokines contribute to the perpetuation of mucosal injuries[15,23] and subsequent ulceration. In the extremely painful ulcerative stage, oral bacteria colonize the ulcer and stimulate surrounding cells to release cytokines and chemokines and produce additional tissue-damaging proinflammatory molecules[23]. The extracellular matrix and submucosal mesenchymal cells interact with innate immune cells to initiate the healing process of mucositis. During this phase, there is proliferation and differentiation of epithelial cells with the reestablishment of oral microbiota[22,23].

Our hypothesis was to quantify the changes undergone by CD4 and CD8 T lymphocytes with and without probiotics during CTRT for LAHNC with the assumption this would throw light on the immunomodulatory mechanisms of probiotics.

Peripheral blood lymphocytes are very sensitive to radiation. Even low-dose total body radiation can reduce circulating lymphocyte counts, which may compromise antitumor immune responses during CRT. In addition to its immunosuppressive effects, CRT can also have immuno-stimulatory that can enhance anti-tumor immune response. Previous studies[24-26] have demonstrated that this immuno-stimulating effect on adaptive immunity can result in a relative increase in the pool of circulating CD4 and CD8 T Lymphocytes at the end of treatment. This corresponded to significantly better survival parameters in their study cohort. Chen et al[24] have observed a rise in the CD8 T cells from 26.1% to 30.6% (p=0.001) in 118 patients of esophageal cancer receiving neoadjuvant chemo-radiotherapy. In our study, a similar response was observed in the CD8 T cell population. There was a consistency in the pattern of increase in the CD8 T cell proportion post CTRT in both study arms as well as the observational arm.

CD8+ T cells are the major anti-cancer effector cells, as they can give rise to cytotoxic T lymphocytes (CTLs) that kill neoplastic cells[26]. CTLs are generated through either the priming of naive T cells or the re-programming of memory T cells. Naive CD8+ T cells differentiate into CTLs in lymphoid organs upon encountering antigen-presenting cells (APCs).

Probiotics are gaining importance as a therapeutic approach for modulating the mucosa-associated immune system. Different strains of probiotics, such as *Lactobacillus acidophillus*, *Lactobacillus reuteri*, and *Bifidobacterium bifidum*, as well as specific strains of *Lactobcaillus rhamnosus*, have shown the capacity to activate dentritic cells and T cells[9-11]. Bowen et al[13] have investigated the use of a probiotic combination of Lactic acid bacteria and bifidobacteria as a preventive treatment for radiation-induced diarrhea. The study involved 490 patients receiving postoperative radiation for cervical, rectal, or sigmoid cancer. Treatment with probiotics significantly reduced the incidence of grade 3 or grade 4 diarrhea as compared to the placebo (1.4% *vs.* 55.4 %, $p < 0.001$). A few trials have also addressed the use of probiotics in the setting of chemotherapy-induced mucositis[27].

Very few studies have addressed the role of probiotics in head and neck chemoradiation. Probiotics are postulated to enhance the healing process by accelerating the re-establishment of the microbial flora. This is probably why grade II mucositis in patients receiving probiotics heal rapidly before they can progress into dose-limiting grade III mucosal reactions. Our study also demonstrated a similar 57% relative reduction in grade III mucositis with probiotics. Jiang et al[4] have analyzed the role of probiotics in the setup of nasopharyngeal carcinoma. Their results indicated that probiotics could significantly reduce oral mucositis, as well as have an impact on the immunity of the patients. Their study showed that only 15.52% of patients receiving probiotics demonstrated grade III mucositis against 45.71% of the cohort receiving placebo (p<0.0001).

An earlier trial by Sharma et al[12] addressed the potential of experimental probiotic lozenges in a cohort of 188 patients with head and neck cancers undergoing chemo irradiation. They observed favorable results in terms of reduction of grade III and grade IV mucositis in patients randomized to receive the trial drug (52%) compared to the placebo arm (77%) ($p < 0.001$). However, this was a trial drug and is not available in the market. The formulation was also limited because it contained only one strain of *Lactobacillus*.

Before analyzing the immune micro-environment alterations in both trial cohorts, we conducted a baseline analysis in an observational cohort of cervical cancer patients. This aimed to determine whether the expected pattern of CD4 and CD8 lymphocyte reduction remained consistent even in the absence of probiotics. This site was chosen based on the assumption that the

substantial inclusion of bone marrow in the radiation field would amplify the impact and pattern of lymphocyte reduction. As anticipated, there was a significant fall in the T lymphocyte counts of about 62%.

The current study confirms the potential of probiotics in drastically reducing the incidence of oral and pharyngeal mucositis and reducing weight loss during radical treatment of head and neck cancers with chemoradiotherapy.

An interesting dichotomy was observed in the pattern of CD4 *vs.* CD8 counts as a result of radiation. There was a paradoxical rise in the CD8 counts *vs.* a reduction in the CD4 counts. The same observation was made in our study, a finding not previously reported in earlier research on the role of probiotics based on current knowledge. Jiang et al[4] observed an increase in both the number of CD4 T cells (76.59% *vs.* 52.85%) as well as CD8 T cells (62.94% *vs.* 29.76%) with probiotics in their study. A larger number of patients would be required to establish the biological correlation of the pattern of the dichotomy concerning mucositis and tumor response-related immunomodulation.

The relative increase in the CD8 that occurs might correlate with the role of probiotics in not only reducing the treatment compromising grade III mucositis but also empowering the body's immune mechanism to achieve better anti-tumoral response. In our study, the CR rates for the experimental arm were almost double (77%) compared to the control arm (33%), supporting this hypothesis.

The major limitation of our study is that we do not have a sufficient number of patients to validate these inferences.

CONCLUSIONS

Our study shows that probiotics can potentially enhance the current standard of care in treating head and neck cancer, i.e., concurrent CTRT. The drastic reduction in the anticipated grade III mucositis might also, directly and indirectly, enhance the chances of a cure. The immunomodulatory potentiation of the CD8 activity in the tumor microenvironment may be a potential mechanism for better tumor control. In other words, probiotics could be synonymous with an affordable, easily tolerated, and accessible immunomodulator for low and middle-income (LMIC) nations.

Conflict of Interests

The authors declare no conflict of interest.

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Authors' Contributions

BJ is responsible for study conception and design, data collection, analysis, and manuscript preparation. NR performed data collection and analysis and manuscript preparation. LV contributed intellectual input during the study conception and design and performed a review and editing of the manuscript. All authors have read and approved the final manuscript.

Availability of Data and Material

All necessary data have been included in the current manuscript. Any further data may be made available upon reasonable request.

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Ethical Approval and Informed Consent to Participate/Publish

This study was performed in line with the principles of the Declaration of Helsinki after obtaining written informed consent from the patients. The study was approved by the ethical committee Kidwai Cancer Institute Medical Ethics Committee of the Kidwai Memorial Institute of Oncology] with protocol number [KCI/MEC/019/14.November.2019 and date 22/11/2019.

Informed Consent

Written informed consent was obtained from all patients before recruitment for the study.

Artificial Intelligence (AI) Disclosure

No AI services have been used for writing any part of this manuscript.

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