JAP&T
TPA-Timișoara

Available online at http://journal-of-agroalimentary.ro

Journal of Agroalimentary Processes and Technologies 2023, *29*(1), 16-22

*Journal of Agroalimentary Processes and Technologies*

# The *in vitro* Toxicological Potential of *Lactobacillus paracasei* on Detroit 562 Pharyngeal Cancer Cells

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***Abstract***

Head and neck cancer (HNC) is a frequent malignancy encountered around the world. The lifestyle, including the consumption of cigarettes and alcohol, represent some of the major causes of this cancer, and the approach of appropriate behavior could significantly improve the situation. The consumption of food supplements is in trend, to balance the intake of nutrients. Probiotics (PBT) are more and more frequently used, due to their multiple properties. The aim of this study was to analyze the effect of *Lactobacillus paracasei* on the Detroit 562 human pharyngeal cancer cell line. The results bring out an important impact, observed in the reduction of cell viability, as well as the reduction of cell confluence. These data highlight the potential of the probiotic on the tested line, however, additional data are needed for the complete usefulness of the PBT in order to use it as a co-treatment in case of pharyngeal cancer.

***Keywords***: probiotics, head and neck cancer, pharyngeal cancer, cytotoxicity, Detroit cells.

## 1.Introduction

Since the late 16th century, the notion of 'alimentation' was more and more frequently encountered in English. In Latin languages, especially in French, 'alimentation' means a combination by which humans produce, prepare, procure, indulge in, share, and digest their foods [1]. Supplementing alimentation with food supplements has become the trend of recent years, aimed to complete the nutritional status. Thus, probiotics (PBT) are some of the frequently used supplements [2].

PBT are live microorganisms that, when taken in certain quantities, confer healthy support on the host. The main benefits are presented in Figure 1. In the last decade, studies started to search how modifications in the gut microbiota composition lead to the disruption of host-homeostasis and if these interactions are associated with conditions that predispose individuals to diseases such as inflammatory bowel diseases, allergies, gastrointestinal disorders, or even cancers [3].

In comparison with the past few decades, when PBT have mainly been studied in the field of the gastrointestinal tract, in recent years, these have begun to be studied closely in the prevention and treatment of different oral diseases, especially in infectious diseases including the PBT's impact on caries, halitosis, periodontal disease, and *Candida albicans* infection. A research highlighted that PBT can prevent and treat dental caries by inhibiting the growth of *Streptococcus mutans* and fighting with them for attachment sites and nutrition [4]. PBT are capable to inhibit periodontal pathogens, to lower the production of sulfide, and to regulate the body's immune function in terms to alleviate periodontal inflammation and halitosis. They can reduce periodontal caries by inhibiting mycelial growth of *Candida albicans* and interfering with its adherence, thus playing an important role in the prevention and reduction of oral candidiasis [5].

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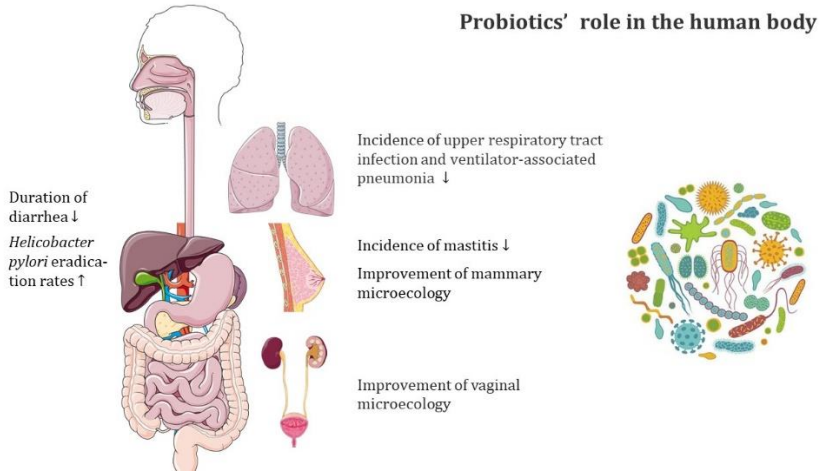


***Figure 1.*** The schematic presentation of the mains roles of probiotics on human health

In addition to caries, inflammation and bacterial contamination, cancerous manifestations at the level of the oral cavity have become subjects of interest, both because of the increase in frequency and the severity of the cases [6]. Oral cavity cancers are included in head and neck cancer (HNC) group, which includes also tumoral formations at the level of pharynx, larynx, salivary glands and paranasal sinuses and nasal cavity. The main causes of this malignancy include: i) alcohol and tobacco use [7], ii) infection with human papillomavirus [8], iii) Epstein-Barr virus infection [9], iv) occupational exposure to different substances (asbestos, nickel, formaldehyde) [10, 11], and v) genetic disorders [12]. Oral hygiene deficient habit has been associated with HNC. Thereby, dental floss use, tooth brushing more than 2/ day, denture wearing, and dental visit ≥1 significantly could reduce the risk of oral cavity cancer, contrary to gum bleeding, missing teeth >5, and periodontal disease, that could increase the risk of oral cavity cancer. For oropharynx cancer type, tooth brushing more than 2/ day and caries surveillance were associated with reduced risk of it. Tooth brushing more than 2/ day and dental visits ≥1 decreased the risk of larynx cancer risk and pharynx cancer risk [13].

The aim of the present work was to analyze the potency of PBT on the Detroit 562 cell line (pharyngeal cancer) in order to complete the plethora of its properties, which could later be associated with the filling and improvement of therapeutic solutions against HNC.

## 2.Materials and methods

### *Materials*

*Lactobacillus paracasei* (CNCM I-1572) was purchased from SofarFarm, dimethyl sulfoxide (DMSO), phosphate saline buffer (PBS), and penicillin/streptomycin were bought from Sigma Aldrich, Merck KgaA (Darmstadt, Germany). MTT (3- (4, 5 – dimethylthiazol – 2 - yl) - 2,5 - diphenyl tetrazolium bromide) kit was achieved from Roche (United Kingdom), trypsin-EDTA solution and fetal bovine serum (FBS) were purchased from Pan Biotech (Aidenbach, Germany). The cell culture media, Eagle's Minimum Essential Medium (EMEM 30-2003) was purchased from ATCC (American Type Cell Collection, Lomianki, Poland). All the reagents were of analytical standard purity and were applied according to the manufacturers' recommendations.

### *Cell Line*

The present study was accomplished using Detroit 562 (CCL-138™) human pharyngeal carcinoma cell lines, which was received as frozen vials. The thawing process started by immersing the vial by gentle agitation in a 37°C water bath (keeping the O-ring and cap out of the water). Then, the water remained on the vial was removed and the vial was decontaminated by spraying with 70% ethanol. The vial's content was gently transferred to a centrifuge tube containing 9.0 mL complete EMEM culture medium, supplied with 10% FBS and 1% penicillin/streptomycin mixture, to avoid contamination, and spin at approximately 125 x g for 5 minutes.

The supernatant was removed, and the cells were resuspended in fresh medium and dispensed into a 25 $cm^2$ culture flask. The culture was incubated at 37°C in an incubator with 5% $CO_2$ and humidified atmosphere. The media was changed every 2-3 days. At 75-80% confluence, cells were transferred in a T75 $cm^2$ flask and after reaching the suitable confluence (80-85%), they were used for the experiment.

*MTT Viability Assay*

The cell viability was assessed by applying the MTT method. Briefly, $10^4$ cells/200 µL/well were cultured in 96-well plates and treated with five different concentrations of PBT (0.5, 10, 50, 100, and 200 million PBT live cells), followed by 24 h of incubation. After the exposure time with PBT, 10 µL/well of MTT solution (5 mg/mL) (kit I) was added and the plate was incubated for 3 h in the incubator, then the formed formazan crystals were dissolved for 30 min in the dark with 100 µL of solubilization buffer (kit II) provided by the manufacturer. At the end, the reduced MTT was spectrophotometrically measured at 570 nm, using the Cytation 5 (BioTek Instruments Inc., Winooski, VT, USA) microplate reader. All experiments were performed in triplicate.

*Cellular Morphology and Confluence Evaluation*

To survey the changes induced by *Lactobacillus paracasei* on confluence and morphology, the pharyngeal carcinoma cells were microscopically examined under bright field illumination and pictures were taken 24 h after treatment with PBT using Cytation 1 (BioTek Instruments Inc., Winooski, VT, USA). The pictures were processed with the aid of the Gen5 Microplate Data Collection and Analysis Software (BioTek Instruments Inc., Winooski, VT, USA). Cell confluence (%) was determined at the end of the treatment using the Image Analysis tool from the Gen5™ Microplate Data Collection and Analysis Software, by succeeding the manufacturer's instructions.

*Statistical Analysis*

All the attendant experimental data are figured as means ± standard deviation (SD). The differences between results were obtained with the GraphPad Prism software, version 10 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com), and compared by means of the one-way ANOVA analysis and Dunett's multiple comparisons post-test. The statistically significant differences between obtained data were labeled with * (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

## 3.Results and Discussion

HNC encompasses a wide array of malignancies arising from the epithelial tissues of the upper aerodigestive tract. It constitutes a significant health burden worldwide, with varying incidence rates among different geographical regions and populations. These tumors are the 7th more frequent encountered form and the 9th cause of cancer death worldwide, with approximately 710,000 incident cases and 359,000 deaths/year [14]. From the incident point of view, oral cavity cancer is the most frequently encountered (Figure 2), from the group of tumors belonging to HNC.

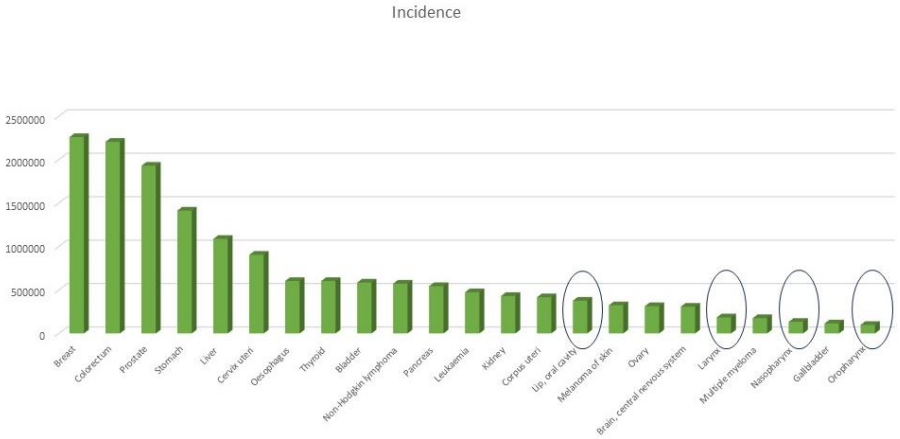


***Figure 2.*** The global incidence rates in the world, both sexes, all ages [15].

***Table 1.*** The in vitro anticancer potential of *Lactobacillus* PBT

| PBT | Cell line | Cancer type | In vitro effect | Source |
|---|---|---|---|---|
| *Lactobacillus paracasei* | HeLa cells | Cervix cancer | ↑ the expression of apoptotic genes BAD, BAX, caspase 3, caspase 8, and caspase 9 and ↓ the expression of the BCl-2 gene. | [24] |
| *Lactobacillus paracasei* | Caco-2 cells | Colon adenocarcinoma | Inhibition of cell proliferation in a time-and dose-dependent fashion. The anti-proliferative effects seems to be mediated through induction of apoptosis by modulating the expression of specific Bcl-2 family proteins. | [25] |
| *Lactobacillus paracasei* | CT 26, HT-29 | Colon carcinoma, colon adenocarcinoma | Attenuation of cell migration, inhibition of cell viability | [26] |
| *Lactobacillus paracasei* | SW-480, HT-29, and HCT-116 | Colon cancer | Blocking the Akt1, mTOR, and Jak-1 mRNAs, and inducing apoptosis by ↓of the anti-apoptotic gene (Bcl-2), ↑ of pro-apoptotic genes (BAX, caspase-3, 8). | [27] |
| *Lactobacillus plantarum* | PC3 cells | Prostate cancer | Lactobacillus facilitated natural killer cell activity by producing tumour necrosis factor-associated apoptosis-inducing ligand, i.e., TNFAIL, in | [28] |
| *Lactobacillus acidophilus* | Tca8113 cells | Tongue cancer | ↓ proliferation of cancerous cells | [29] |
| *Lactobacillus* strains | KG-1 | Leukemia cells | ↑ apoptosis in cancer cells in a dose-dependent fashion, ↓mitochondrial polarization. | [30]. |

The pathogenesis of head and neck cancer involves a complex interplay of molecular events. Chronic exposure to carcinogens, such as those found in tobacco and alcohol, leads to genetic and epigenetic alterations that drive tumorigenesis [16].

Additionally, HPV-associated HNCs show distinct molecular pathways, including the inactivation of p53 and retinoblastoma (Rb) tumor suppressor genes, which contribute to cell cycle dysregulation and increased proliferation [8]. Dysregulation of various signaling pathways, including EGFR, PI3K/AKT, and MAPK, plays a crucial role in promoting tumor growth and resistance to therapy [17].

Early detection of HNC is pivotal for optimal patient outcomes.

Diagnostic methods include clinical examination, endoscopy, imaging techniques (computed tomography, magnetic resonance imaging, and positron emission tomography), and biopsy for histological confirmation. Emerging non-invasive approaches, such as liquid biopsy and biomarker analysis, show promise in improving early detection rates and monitoring treatment response [18]. The management of tese tumors requires a multidisciplinary approach.

Treatment modalities include surgery, chemotherapy, radiation therapy, targeted therapy, and immunotherapy [18].

The choice of treatment depends on tumor stage, site, and patient characteristics. The most common chemotherapy drugs used include cisplatin [19], cetuximab [20], oxaliplatin [21], nedaplatin [22], and the monoclonal antibody nimotuzumab [23]. The disadvantage of these medicines is the lack of selectivity and the high toxicity. Thereby, the aim of the present work is to observe the behavior of *Lactobacillus paracasei* PBT on Detroit 562 cells, to identify possible potential against HNC.

The selection of PBT from *Lactobacillus* group for this experiment was done due to it's various in vitro potential in the cancer field (Table 1).

Following the MTT assay, it was observed that PBT downregulated cell viability, beginig with the lowest concentration. When compared to control, viability was reduced with 16.5%, and the trend was maintened at all tested concentrations up to 30% viability reduction (in case of 100 million live PBT cells treatment - Figure 2).

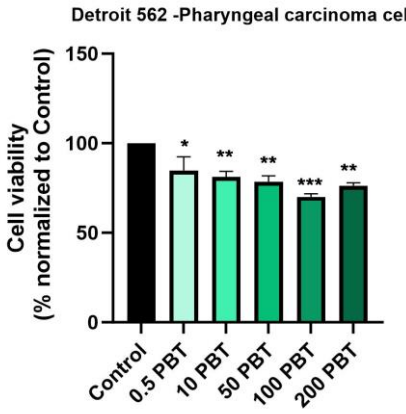


***Figure 2.*** In vitro assessment of the effect PBT (0.5, 10, 50, 100 and 200 million live PBT cells) exerts on the viability of Detroit 562 cells after 24 h of treatment by applying the MTT assay. The data are presented as viability percentages (%) normalized to untreated cells and expressed as means ± SD of three independent experiments. The statistical differences were identified by applying the one-way ANOVA analysis followed by the Dunett's multiple comparisons post-test (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$).

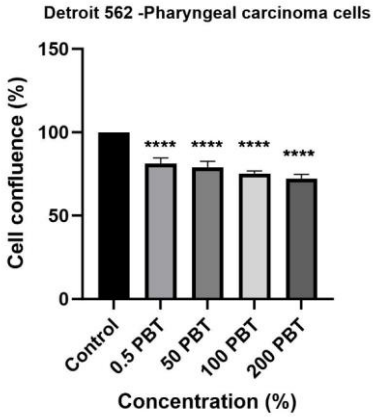


***Figure 3.*** Graphical representation of the calculated cell confluence (%) of Detroit 562 cells following the 24 h treatment with 0.5, 50, 100 and 200 million live PBT cells. The statistical differences between control and the treated groups were identified by applying the one-way ANOVA analysis followed by the Dunett's multiple comparisons post-test (**** $p < 0.0001$).

Similar results were obtained on Caco-2 cells treated with 100 µg $ml^{-1}$ of *Lactobacillus paracasei* for 72 h, when the viability was reduced with 40% [31]. The results agreed also with those reported from Kim et al. [32] that peptidoglycans derived from lactic acid bacteria at a concentration of 100 µg $mL^{-1}$ had significant antiproliferative activity and from Fichera and his collaborator [33] that peptidoglycan from *Lactobacillus casei* at different doses decreased the viability of leukemia and breast tumor cell lines by 25–30%.

The next step of the present work was the analysis of morphological and confluence changes. Thus, cells were observed after 24h of treatment with 4 concentrations (10 million PBT concentration was eliminated because of the similar data obtained with 50 million live PBT cells, in case of the viability test), and compared with control (untreated cells). Results are presented in Figure 3.

It was observed a significant reduction in confluence (with 20%, compared to untreated cells), instead, the cell shape does not seem to be significantly affected. Similar observations were obtained in a study, where cellular changes were observed after treatment with *Lactobacillus sporogenes* and *Clostridium butyricum*, which induced significant cell morphological alterations at all 24, 48 and 72h intervals, but especially after 72 h of treatment [34].

In another research it was observed that cell-free supernatants from *Lactobacillus casei* and *Lactobacillus rhamnosus* have been able to prevent colon cancer cell invasion suggesting that PBT has anti-metastatic bioactive potential [35]. Such downregulation in cell invasion has been later associated with a downregulation in matrix metalloproteinase-9 protein level in cultured metastatic human colorectal carcinoma cells and an upregulation in the level of the tight junction protein ZO-1 [36].

## 4.Conclusions

HNC represent an actual global problem, and the existing current treatments have gaps, so the identification of safe solutions is necessary. The present work proposed to analyze the effect of PBT on the Detroit 562 cancer line, in terms of cell viability and confluence. It was observed a reduction on cell viability up to 30% when treated with 100 million live PBT cells of *Lactobacillus paracasei* strains and a 20% confluence alleviation. These findings need supplementary investigations for PBT as a potential role in the treatment option in case of pharyngeal cancer.

**Compliance with Ethics Requirements.** Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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