Antiproliferative effect of *Lactobacillus casei* and *Lactobacillus fermentum* cell-free supernatants on colonic cancer cell line

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Abstract

Cell-free supernatants of two probiotics namely *Lactobacillus casei* and Lactobacillus fermentum were assessed for their antiproliferative and antitumor activity on human colon cancer CaCo2 and normal NCM425 cell lines. MTT 3-(4,5- Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was used to detect cytotoxic activity and cell viability. Our results indicated that both supernatants exhibited cytotoxic activities and their impact was in a dose-dependent manner. The results also showed that the supernatants were less toxic to normal cells. Lactobacillus casei cell-free supernatant (LcCFS) was more cytotoxic on cancer cells than Lactobacillus fermentum cell-free supernatant (LfCFS) at higher concentrations. However, no significant differences were observed between the impact of LcCFS and LfCFS on the NCM425 cell line. In conclusion, cell-free supernatants of Lactobacillus casei and Lactobacillus fermentum could be good candidates for cancer treatment and control.

Keywords: *Anticancer activity, Lactobacillus casei, Lactobacillus fermentum, cytotoxicity, colon cancer.*

1. Introduction

Probiotics are essential microbes in a balanced human microbiome (Roy and Trinchieri, 2017). The most prevalent probiotics are *Lactobacillus* species. Lactic acid bacteria (LAB) have a variety of health-promoting properties, including the reduction of allergy responses, along with anti-inflammatory and anti-tumor properties (Ding *et al.*, 2018; Kahouli *et al.*, 2017; El-Deeb *et al.*, 2018; Tukenmez *et al.*, 2019). The vast bulk of anticancer research focuses on colon cancer since it is the world's third most prevalent cancer kind (Siegel *et al.*, 2018). In colon cancer cells, *L. casei* has been demonstrated to exhibit anti-proliferative, pro-apoptotic, and anti-tumor properties (Tiptiri-Kourpeti *et al.*, 2016). Another study found that *L. casei*-derived ferrichrome inhibits tumor growth by activating the JNK signaling system (Konishi *et al.*, 2016). *Lactobacillus acidophilus*, according to another research, makes colorectal cancer cells more susceptible to 5-fluorouracil-induced death (Baldwin *et al.*, 2010). Zitvogel *et al.*, (2017) mentioned that probiotics produce particular chemicals that trigger anti-tumorigenic molecules to target cancer cells. *Lactobacillus acidophilus* 606 soluble polysaccharides were shown to have anticancer potential and to cause apoptosis in HT-29 cells (Choi *et al.*,2006). Cell-free supernatants from the probiotic *Lactobacillus casei*, according to (Escamilla *et al.*, 2012), reduce colorectal cancer cell migration *in vitro*. Furthermore, *L. fermentum* had been shown to inhibit angiogenesis and tumor migration in colon cancer (Liu *et al.*, 2021).

Colon cancer is becoming more common as people's dietary patterns change (Clerici *et al.*, 2021). Colon cancer is one of the most fatal tumors due to its high metastasis and aggressive nature. The progression of

colorectal cancer is a multistep process (Clerici *et al.*, 2021). The buildup of mutations in specific tumor suppressor genes and protooncogenes may result at the beginning of cancer (Liu *et al.*, 2011). Clinical studies have indicated that people with gastrointestinal illnesses frequently have intestinal flora abnormalities (Girardin and Seidman, 2011). The intestinal microbial community and interactions between the host and bacteria might be crucial factors in colorectal cancer (Liu *et al.*, 2021).

The objective of the present study was to explore the effects of cell-free supernatant of *L. casei* and *L. fermentum* on the proliferation of human colon cancer cells CaCo2 as well as normal colonic cell NCM425.

2. Materials and Methods

2.1. Bacterial growth conditions and cell-free supernatant preparation:

Probiotic isolates were obtained from the Department of Biology/College of Science/ Al Mustansiriyah University. They were activated twice by subculturing its stock solution in MRS broth (Oxoid, UK) with incubation anaerobically at 37 ˚C for 24 and 48 hrs. To prepare cell-free supernatant (CFS), 120 ml of MRS broth containing $1x10^8$ CFU/ml of 24 h grown *L. casei* and *L. fermentum* culture were incubated anaerobically, for 72 h. at 37 ˚C, then centrifuged at 4000 rpm for 15 m and filtered throughout 0.22 µm pore-size filter papers (Microlab, UK) before lyophilizing and keeping at -20 ˚C till use.

2.2. Cell line maintenance and MTT assay:

Five concentrations (50,25,12.5,6.25, and 3.1 µg /ml) of the CFS of each of *L. casei* and *L. fermentum* were prepared after adding 0.5 ml of distilled water to the lyophilized product, and by using an MTT kit (Intron Ltd kit, China) which was tested upon CaCo-2 cell culture and NCM425. Each concentration was cultured on the medium of RPMI1640; the cells were then removed by EDTA/trypsin solution and resuspended in a medium containing 10^{-1} percent bovine serum albumin and then plated on a 96-well microtiter plate. (All steps were done in triplicate). The MTT assay was used to examine the anticancer potential activity, and the results were read at 517 nm after 24 hrs. The following formula was used to calculate the cell viability:

%Viability = Mean OD sample / Mean OD blank ×100.

2.3.Statistical analysis:

Data analysis was carried out by ANOVA test and a *P-value* of < 0.05 was determined to be statistically significant. GraphPad Prism version 8.4.3 was used for all statistical analyses.

3. Results and Discussion

3.1. Cultural and microscopic characteristics of L. casei and L. fermentum:

Gram-positive bacilli were seen under the microscope, mostly in chains. They did not develop endospores and were nonmotile, isolates formed spherical, very light yellow or creamy white colonies when cultivated on MRS media which were similar to the findings of (Ahmad *et al.*, 2018).

3.2. Biochemical characterization of *L. casei* and *L. fermentum*:

L. casei and *L. fermentum* gave catalase and oxidase-negative results when there was no bubbles formation occurred nor change in color to blue after adding oxidase reagent, which came by what was stated in (Angelescu *et al.*, 2019).

3.3. Antitumor activity of cell-free supernatant of Lactobacillus spp.:

i. **Antitumor activity of *L. casei* cell-free supernatant (LcCFS):**

The half maximal inhibitory concentration (IC50) values Figure-1 demonstrated that LcCFS had a more cytotoxic impact on cancer cell lines than on normal cells, (54.78, 165.70) µg/ml on CaCo-2 and NCM425

respectively. This could be attributed to the fact that lactic acid bacteria exhibit little or no cytotoxicity on normal cells since they generate selective growth inhibitors, which specifically target cancer cells (Choi *et al.*, 2006). Tiptiri-Kourpeti *et al.*, (2016) observed a similar conclusion of *Lactobacillus casei* (live and bacterial components) that displayed a significant dose- and time-dependent antiproliferative impact on human cell lines from colon cancer. Furthermore, prior research found that *L. casei* cell-free supernatants had anti-cancer effects, such as increasing apoptotic genes (BAX, BAD, caspase 9, caspase 8, and caspase3) and decreasing BCL-2 (Shahid *et al.*, 2018). Likewise, the ability of *L. casei* exopolysaccharide to induce anticancer activity in human colon cancer cell lines was revealed in research (Liu *et al.*, 2011), while normal intestinal epithelial cell viability was unaffected.

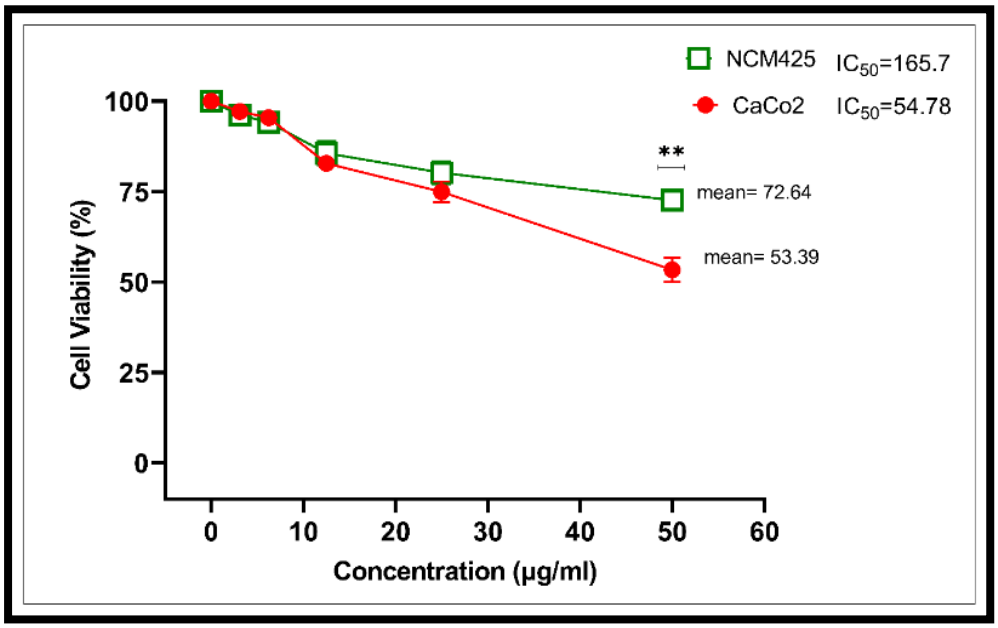

Figure 1. Cytotoxic activity of Lactobacillus casei cell-free supernatant (LcCFS) on CaCo2 and NCM425 cell lines

ii. **Antitumor activity of *L. fermentum* cell-free supernatant (LfCFS):**

The observations of *Lactobacillus fermentum* cell-free supernatant (LfCFS) on cell lines revealed that LfCFS has cytotoxic action on the cells examined in a dose-dependent manner, with the largest impact found at 50 µg/ml, inhibiting CaCo-2 and NCM425 viability Figure-2. The LfCFS was more cytotoxic to cancer cell lines than normal epithelial cells, as evidenced by statistical analysis, which revealed a significant difference between the two cell lines at all doses. The IC_{50} values of LfCFS had greater cytotoxicity on CaCo-2, as evidenced by an IC_{50} of 74.67 µg/ml, which is lower than the 210 µg/ml on NCM425 Figure-3. *L. fermentum* cell-free supernatant was discovered to have anticancer potential in research that looked at cytotoxicity in 3D culture systems against colorectal cancer (Lee *et al.*, 2019). *L. fermentum* RM28 also inhibits the proliferation of colon cancer cells in a study (Kahouli *et al.*, 2017).

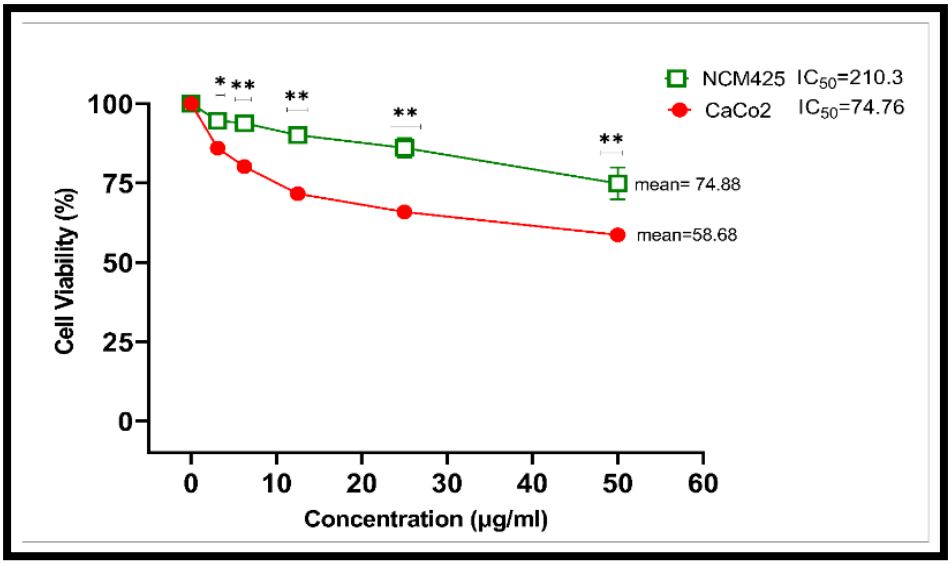

Figure 2. Cytotoxic activity of Lactobacillus fermentum cell-free supernatant (LfCFS) on CaCo2 and NCM425 cell lines

The antiproliferative activities of *L. fermentum* on colon cancer cells help minimize the risk of colon cancer development (Liverani *et al.,* 2019). Earlier studies reported that autophagy initiated by *L. fermentum* in acetaminophen (APAP) increased cytotoxicity in HepG2 cells (Dinić *et al.*, 2017). Furthermore, thecolonic normal cell line was not affected when treated with *L. fermentum* cell-free supernatant as stated by (Kahouli *et al.*, 2017).

iii. Comparison between the antitumor activity of cell-free supernatant of *L. casei* and *L. fermentum*:

When a comparison was made between the cell-free supernatant effect of the two probiotics results showed that LcCFS was more cytotoxic on CaCo2 which was evident by statistical analysis when the *p-value* was < 0.05 at higher concentrations Figur-3 while no significant differences between LcCFS and LfCFS at lower concentrations were recorded. A study (Wang *et al.,* 2012) showed that cell wall extracts of locally isolated *L. casei* have ahigher inhibitory rate than other probiotics namely *L. casie* 53103, *L. paracasei subp. paracasei* and *L. rhamnosus* when tested for antiproliferative effect on HT-29 cells after 72 h while *L. casie* 53103 was more effective when cells were treated for 48 h. Another study demonstrated that *L. casei* presented better characteristics in terms of antagonistic materials production when compared to other strains of probiotics (Martins *et al.*, 2009). However, Peran *et al.*, (2007) mentioned that *L. fermentum* was more effective than *L. reuteri* in reducing colon cancer cell growth when tested in rat models. It has been proven that *L. fermentum* has shown more anticancer potential among other lactobacilli species (Mokhtari *et al.*, 2021). On the other hand, differences between the impacts of LcCFS and LfCFS on normal cell line NCM425 weren't observed despite the fact that LfCFS showed a slightly safer impact than LcCFS but it was not significant at a p-value < 0.05 Figure-4. In this regard, (Choi, 2006) pointed out that heat-killed *L. casei* ATCC 393 was more toxic on normal healthy human embryo fibroblasts (cells) than heat-killed *L. acidophilus*

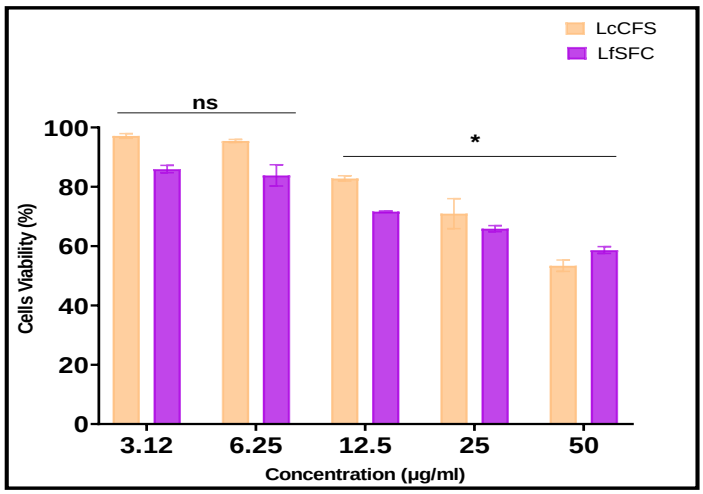

Figure 3. Comparison between Lactobacillus casei cell-free supernatant (LcCFS) and Lactobacillus fermentum cell-free supernatant (LfCFS) on CaCo2

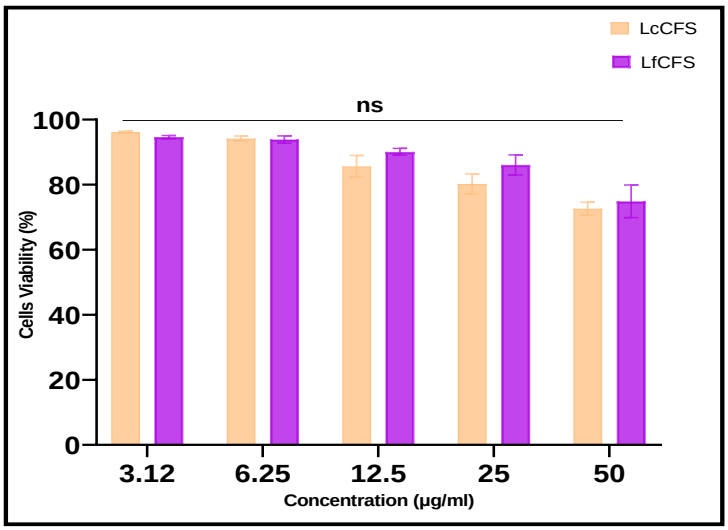

Figure 4. Comparison between Lactobacillus casei cell-free supernatant (LcCFS) and Lactobacillus fermentum cell-free supernatant (LfCFS) on NCM425

Conclusions

It is noteworthy that cell-free supernatants of *Lactobacillus casei* and *Lactobacillus fermentum* were cytotoxic on human colon cancer cells with lesser cytotoxicity on non-cancerous cells which may propose an alternative promising anticancer therapy.

Conflicts of Interest

The authors declare no conflicts of interest

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التأثير المضاد للتكاثر للراشحين الخاليين من الخلايا لـ *Lactobacillus casei* و*Lactobacillus fermentum* ضد خط خلايا سرطان القولون

مصطفى عطية حديد: قسم طب الاسنان/ كلية الفارابي الجامعة/ بغداد - العراق
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محمد فرحان الحلبوسي: مركز بحوث التقنيات الاحيائية/ جامعة النهرين/ بغداد - العراق

الخلاصة

تم فحص فعاليتي مضاد التكاثر ومضاد السرطان لراشحي المعززات الحيوية الخالي من الخلايا Lactobacillus casei (LcCFS) و Lactobacillus fermentum (LfCFS) على خطي خلايا سرطان القولون Caco-2 و الخلايا الطبيعية NCM425. تم استخدام فحص الMTT لقياس الفعالية السمية الخلوية و حيوية الخلايا. أظهرت النتائج ان كيلا الراشحين يحتويان على فعالية سمية خلوية و كان التأثير معتمدا على التركيز. كما أظهرت النتائج ان الراشحين اقل سمية على الخلايا الطبيعية. راشح Lb. casei (LcCFS) كان اكثر سمية خلوية على خط الخلايا السرطانية مقارنة مع راشح Lb. fermentum (LfCFS) . على الرغم من ذلك, لم يلاحظ فرق معنوي بين تأثير LcCFS و LfCFS على خط خلايا NCM425. يستنتج من ذلك ان الراشحين الخلويان لل LcCFS و LfCFS يمكنهما ان يكونا مرشحان جيدان لمعالجة السرطان و السيطرة عليه.