Effect of the postbiotic produced by Bacillus mojavensis from pulque on colon cancer cells

María Alejandra Trujillo-Lopéz
Autonomous Popular University of Puebla State: Universidad Popular Autonoma del Estado de Puebla

Miguel Ángel Garduño-Vargas
Autonomous Popular University of Puebla State: Universidad Popular Autonoma del Estado de Puebla

Elizabeth Bautista-Rodríguez (elizabeth.bautista@upaep.mx)
Universidad Popular Autónoma del Estado de Puebla  https://orcid.org/0000-0003-0410-0415

Cristina Muñoz-Olivos
Autonomous Popular University of Puebla State: Universidad Popular Autonoma del Estado de Puebla

Verónica Miroslava Martínez-Ortiz
Autonomous Popular University of Puebla State: Universidad Popular Autonoma del Estado de Puebla

Beatriz Pérez-Armendáriz
Autonomous Popular University of Puebla State: Universidad Popular Autonoma del Estado de Puebla

Elie Girgis El-Kassis
Autonomous Popular University of Puebla State: Universidad Popular Autonoma del Estado de Puebla

Research Article

Keywords: Bacillus mojavensis, postbiotics, colon cancer cells, Pulque

Posted Date: October 6th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3338439/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Probiotics have shown their potential antiproliferative effect on colon cancer cells. Although studies suggest that the effect is due to microbiome regulation, it also could be caused by metabolites produced by probiotics. Therefore, in the present work, we evaluated the effect of a cell-free extract called postbiotic from *B. mojavensis*, a strain isolated from Pulque (a traditional Mexican drink); the viability was determined by the MTT and crystal violet assays and the migration using wound-healing assay on the colon cancer cell SW-480. In addition, the effect on Human Peripheral Blood Mononuclear Cells (PBMCs) obtained from healthy patients was determined. The results showed that 25 or 50 µg/ml of *B. mojavensis* decreased viability in more than 75% of SW-480 cells and migration after 24h. Moreover, 25 or 50 µg/ml of *B. mojavensis* showed the ability to inhibit LPS-induced proliferation by 10ug/mL. In addition, interestingly, the effect is specific for colon cancer cells since *B. mojavensis* did not induce a cytotoxic effect on healthy PBMCs cells. The reported results show that the postbiotic produced by the studied strain of *B. mojavensis* isolated from pulque has excellent potential use as an adjuvant in treating colorectal cancer.

Key points

*B. mojavensis* isolated from Pulque produces postbiotic that induce cytotoxicity.

Postbiotic inhibits migration on colon cancer cells.

Postbiotic decreases LPS-stimulated proliferation in colon cancer cells.

Introduction

Colorectal cancer (CRC) represents a major global cause of cancer mortality, being the third most common cancer worldwide, with a projected continuous increase mainly in high or very high Human Development Index (HDI) countries (Morgan et al. 2023). The prognosis of survival depends on the stage of the disease; patients with stage I CRC have a 90 percent survival prognosis, while patients with stage III are predicted to have a 5-year survival, and patients with stage IV display only a 10% percent survival expectancy (Chen et al. 2019; Van der Jeught et al. 2018). The first line of treatments consists of surgery, targeted therapy, radio, and chemotherapy; however, drug resistance is one of the main difficulties when treating this cancer type (Van der Jeught et al. 2018) Increasing evidence has shown a correlation between microbiome alteration, also called dysbiosis, and CRC disease progression (Ahn et al. 2013; Arthur et al. 2012; Kostic et al. 2013; Sobhani et al. 2011; Yang et al. 2021).

On the other side, it has also been found that some microbiota metabolites from healthy donors repress cancer growth and metastasis (Bell et al. 2022) Therefore, the gut microbiome and its metabolites have been used for cancer prevention and as potential treatment adjuvants (Van der Jeught et al. 2018; Saeed et al. 2022). Probiotics have been widely used for the treatment of different diseases, including the CRC, due to their biological effects: they improve the intestinal barrier function, regulate the gut microbiome
composition, secrete anticancer molecules, and degrade some carcinogenic compounds (Tripathy et al. 2021) and in recent years the use of postbiotics for cancer therapies has growth. According to the International Scientific Association for Probiotics and Prebiotics (ISAPP): “Postbiotics is a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host” (Salminen et al. 2021). High levels of secondary bile acids and SCFAs (short chain fatty acids) secreted by the microbiota are linked with lower colon inflammation (Song et al. 2020), and metabolites like reuterin, produced by *Lactobacillus reuteri* modify the redox balance and reduce proliferation and survival in colon cancer cells inhibiting ribosomal biogenesis and protein translation (Bell et al. 2022).

On the other hand, the anticancer effects of probiotics and much less of their postbiotics of the *Bacillus* genus have been little explored despite their multiple advantages over *Lactobacillus* (Song et al. 2023). Due to its sporulation capacity, the *Bacillus* has greater viability since it is resistant to the drying process, is tolerant of temperature changes, and survives at low pH, thus resisting gastrointestinal conditions, allowing it to reach the intestine (Mingmongkolchaisri and Panbangred 2018). In this regard, the workgroup has explored the probiotic potential of the *Bacillus mojavensis* strain isolated from aguamiel (traditional Mexican drink) called UPAEP-B-F2a, which has been confirmed by microscopic identification, mass spectroscopy, 16srRNA sequencing, and biochemical profile. Also, its antagonistic effect on multi-resistant pathogenic bacteria has been verified as a probiotic and postbiotic (Martínez-Ortiz et al., unpublished results). Therefore, in this work, we analyze the effect of the postbiotics produced by a strain of *Bacillus mojavensis* on the viability stimulated with or without LPS and migration of SW-480 colon cancer cells. Additionally, we evaluated its specific effect on cancer cells by determining its effect on Human Peripheral Blood Mononuclear Cells (PBMCs).

**Material and methods**

**Bacterial culture and Postbiotic (CFS)**

To generate a pre-inoculum, *B. mojavensis* (WFCC-CDBB-500 with No. B-2077) was cultivated in MRS medium (De Man, Rogosa and Sharpe) for 24 hours at 37°C. Later, the optimum density was adjusted to 0.5 McFarland to have 108 bacteria. The culture was carried out at 2% in MRS broth from this growth for 48h at 37°C. Once the time was over, the cell package was separated by centrifuging at 4500 rpm for 10 min at 4°C to recover the cell-free supernatant, also called postbiotics which was subsequently filtered using a 0.22 µm PES membrane.

**Cell culture**

SW480 colon cancer cells were cultured in DMEM-F12 medium (Dulbecco's minimum essential medium; Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin (Life Technologies, Carlsbad CA), and maintained in a humidified 5% CO2 and 37°C ambiance. Subsequently, the amount of proteins present in the postbiotic was quantified by the Bradford method (Sigma Aldrich, St Louis, MO).
MTT assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Sigma Aldrich, St Louis, MO). A total of 40,000 SW480 cells were seeded in 48-well plates and subsequently treated with 50 µg/mL and 25 µg/mL of the *B. mojavensis* postbiotic for 24h. At the end of the time, the cells were incubated with MTT at a concentration of 0.5 mg/mL. The medium was removed for 2 h at 37°C in the dark, and the formazan crystals were dissolved with 200µl of dimethylsulfoxide (DMSO). The absorbance was measured using a plate reader (Thermo Scientific, Multiskan FC) at a length of 540 nm. All experiments were performed in triplicate, and the data were normalized with respect to the absorbance measured in control cells and are shown in percent viability.

Crystal violet assay

SW480 cells were seeded 40,000 cells per well in 48-well plates and were maintained in a humidified 5% CO2 and 37°C ambiance. After 24h, they were treated with MRS medium as a control, 50 µg/mL or 25 µg/mL of post-inhibition for 24 hours. At the end of the time, the culture medium was removed, and the cells were washed 3 times with 200µl of phosphate buffer saline (PBS). They were then fixed with 200µl of 4% paraformaldehyde for 5 minutes and then again washed 3 times with 200µl of PBS and 100µl of 0.05% crystal violet (CV) solution in 20% ethanol added for 1 minute and then washed with PBS and observed under an inverted microscope.

Wound healing test

SW480 cells were seeded in 24-well plates with 20,000 cells/well. After 24h, a sterile pipette tip was used to make the wound linearly to eliminate the cell monolayer, the culture medium was removed, and the MRS treatments, 50 µg/mL and 25 µg/mL of postbiotic, also microphotographs were taken in bright field. After 24h with the treatment, the cells were stained with crystal violet and observed under a microscope.

LPS treatment

SW480 cells were seeded in 48-well plates at 40,000 cells/well. They were then treated with 10 µg/mL of *Escherichia coli* O111:B4 lipopolysaccharide (L2630-25 Sigma Aldrich, St Louis, MO) and with 50 µg/mL or 25 µg/mL of *B. mojavensis* postbiotic for 24 h. Subsequently, cell viability was evaluated by MTT.

Obtaining PBMCs and postbiotic treatment.

PBMC were isolated from 5 ml of human venous blood using lymphoprep according to the manufacturer's instructions (Sigma Cat:C4-039391/03). Once the PBMCs were obtained, they were cultured in RPMI medium supplemented with 10% SFB, 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin (Life Technologies, Carlsbad CA), and maintained in a humidified 5% CO2 and 37°C ambiance. For postbiotic treatment of *B. mojavensis* PBMCs were seeded 40,000 cells per well and treated with 30 or 50% v/v postbiotic. After 24h of incubation, viability was evaluated by the MTT assay.
Statistical analysis

Results are expressed as means ± SEM. Before statistical analysis, all data were tested for normality and equality of variances. ANOVA tests were used for multiple comparison analysis, followed by Dunnet's post hoc to evaluate differences between groups. The analyzes were performed using the statistical program Graph Prism version 8.0.

Results

Bacillus Mojavensis postbiotic induces cytotoxicity of SW480 colon cancer cells.

Colorectal cancer cells were incubated with MRS medium as control or 25 or 50 ug/mL of the postbiotic of B. mojavensis. After 24 h using the MTT technique, a decrease in cell viability was observed in the groups treated with B. Mojavensis with a ****p < 0.001 versus control, and with no significant difference between the two of them (Fig. 1a). The decrease in cell number was corroborated using crystal violet staining (Fig. 1b).

Bacillus Mojavensis Postbiotic inhibits migration of SW480 colon cancer cells.

In SW-480 colon cancer cells, the Toll-like receptor 4 (TLR4) expression has been reported, which participates in the induction of the inflammatory process. TLR4 is a receptor stimulated by LPS, which is produced by Gram-negative bacteria such as E. Coli (Rakhesh et al., 2012; Zhu et al., 2019; Mempin et al., 2021). In this regard, it has been reported that the inflammatory process induced by LPS promotes the proliferation of SW-480 colon cancer cells. Therefore, in this work, we induced the proliferation of SW-480 cells with 10µg/mL of LPS for 24 h; the results showed an increase in viability of more than four times with respect to the control. Interestingly, co-incubation with the postbiotic B. mojavensis reversed the increase in cell viability induced by LPS by up to 375.68%.

We performed the wound assay and staining with crystal violet to investigate the role of B. Mojavensis postbiotic on the migration of SW-480 cells. The results show that the postbiotic at 25 and 50 µg/mL concentrations after 24 h strongly inhibits SW-480 colon cancer cell migration (Fig. 3).

Bacillus Mojavensis postbiotic does not induce cytotoxicity on Human Peripheral Blood Mononuclear Cells (PBMCs).

We performed the culture of PBMCs from healthy patients, and the cells were incubated in RPMI medium as control, MRS, or the postbiotic of B.mojavensis in a final volume of 30% (v/v) in RPMI medium respectively during 24 h. Also, we found no significant difference between the MRS or postbiotic conditions compared to the control; however, the co-incubation with the postbiotic increased the viability compared with the MRS condition with a ****p < 0.0001 (Fig. 4).
Discussion

The development of CRC involves a complex interaction between intrinsic and extrinsic factors, including gut dysbiosis. In this regard, mainly opportunistic pathogenic bacteria that produce enterotoxins such as fraglysin, colibactin, and cytolethal distension toxin have been identified in CRC (Zhou and Fang 2018), which contributes to the inflammatory process and the "driver-passenger" effect. This implies that bacteria called drivers colonize in the first step and invade, causing damage for that; in the second step, it allows other commensals called passengers or their products to pass through the epithelium to enhance the damage (Avril and DePaolo 2021). Thus, in a first approach, probiotics, mainly Lactobacillus, have been proposed to be an excellent option to restore the imbalance; though this is a proposed mechanism of action by modulating bacterial populations, it could also be cause of postbiotics, that is, the products or byproducts, metabolic secreted by living probiotic bacteria or released after bacterial lysis such as bacteriocins, biosurfactants, exopolysaccharides, and siderophores, among others (Kanmani et al. 2013). The Bacillus genus has also been shown to have anticarcinogenic properties. In fact, the supernatant of B. coagulans overexpresses the pro-apoptotic genes bax, casp3, and casp9 in SKBR3 breast cancer cells (Dolati et al. 2022); consequently, the Bacillus are potential targets. study around the CRC.

Thus, this work demonstrated that the postbiotic of B. Mojavensis isolated from Pulque decreases the viability and migration of colorectal cancer SW-480 cells, reinforcing the benefits of the metabolites produced by probiotics. As probiotics, Bacillus species are beginning to be studied in their vegetative and spore forms because the latter has excellent stability under different environmental and gastrointestinal conditions and can germinate in the gastrointestinal tract, colonize, and generate stimulation of the immune system more efficiently than vegetative cells, the most studied being B. subtilis, B. clausii, B. coagulans, B. licheniformis, and B. polyfermenticus (Duc et al. 2004; Lee et al. 2019).

Chen et al, (2015) demonstrated that the conditioned medium of B. subtilis participates in the protection against damage in intestinal epithelial cells and inhibits the growth of colorectal cancer cells in a time and dose-dependent manner, promoting apoptosis and cell cycle arrest. In addition, in mice treated with dimethyldrazine (DMH) supplemented with B. subtilis, the expression of Th2 and Th17 increased by DMH was reduced, as well as the expression of transcriptional factors associated with the inflammatory process and cancer progression such as TLR4–MYD88–NF-κB, IL-22, and SURVIVIN, as well as increased expression of the cyclin-dependent kinase inhibitor p21 and decreased levels of NF-κB, p-ERK, and β-catenin (Chen et al., 2015)(Chen et al., 2015).

The B. Mojavensis postbiotic used in this work is undergoing metabolomic and proteomic analysis. However, some metabolites produced by Bacillus with anticancer properties have recently been reported. For example, a cytotoxic compound was isolated from B. vallismortis strain BIT 33, which has been shown to have high cytotoxicity and proapoptotic capacity at 1 and 10 µg/mL in colon cancer cell lines HCT116, HT-29, and SW480 (Jeong et al. 2008).

The inhibition of the migration is an anti-cancer property of probiotics such as Lactobacillus and Bifidobacterium represent a therapeutic option for metastasis (Motevaseli et al. 2017). For example,
administering a pool of *B. longum*, *B. bifidum*, *L. acidophilus* and *L. plantarum* has shown the ability to inhibit migration, CT26 cell invasion, and tumor growth (Shang et al. 2020). Interestingly, we now show that the postbiotic of the used strain of *B. mojavensis* isolated from pulque inhibits cancer cell migration. The mechanisms by which they do so are not fully described. However, it has been reported that the supernatant of *L. acidophilus* and *L. rhamnosus* GG decreases the expression of extracellular matrix metalloproteases such as MMP2 and MMP9 and increases the expression of metallopeptidase inhibitors such as TIMP-1 and TIMP-2 in HeLa and HT29 cells (Nouri et al. 2016). TIMP-1 has been identified as a critical protein in colon cancer since it promotes cell proliferation and invasion capacity (Ma et al. 2022). Of the metabolites responsible for the inhibition of migration, the group of An et al. (2019) reported that an 8 kDa protein produced by *Lactobacillus rhamnosus* KCTC 12202BP inhibited the migration of DLD1 colon cancer cells and further down-regulated expression of Cyclin B1 and Cdk1.

The chronic inflammatory process is a factor that favors the progression of cancer of the esophagus, mouth (Gonçalves et al. 2016), gastric (Chochi et al. 2008), bladder (Olbert et al. 2015), and CRC (ZHU et al. 2019). In this context, it has been identified that inflammation can be induced by bacterial LPS, which has been reported to induce increased expression of TNF-α, IL-6, COX-2, MMP-7, and VEGF-C in the SW480 colorectal cancer cell line. This effect is abolished when the expression of MyD88 is suppressed, which induces the inhibition of the expression of /NF-κB/MAPK (Zhu et al., 2019). In contrast, anti-inflammatory effects of probiotic bacteria such as *Bacillus polyfermenticus* KU3 and *Lactococcus lactis* NK34 have been demonstrated, which decrease the levels of proinflammatory cytokines induced by LPS (Lee et al. 2015). In this work, we report that LPS effectively induces the proliferation of SW-480 cells, and we show for the first time that this effect is suppressed by the *B. Mojavensis* postbiotic, evidencing its potential use in the treatment of colorectal cancer.

Regarding the molecules present in postbiotics, it has been shown that the extracellular polymeric substance (EPS) produced by *B. subtilis* at a concentration of 400 g/mL has been shown to decrease HeLa cell viability after 24 hours. In addition, the oral administration of 200 mg/kg of EPS by oral administration increased the phagocytic activity of macrophages and the levels of IL-2 in the blood. The mechanism of action of EPS has not been elucidated, however, it has antioxidant activity (Zhang and Yi 2022). Por otro lado, *B. subtilis* cuando se cultiva en medio Difco Sporulation Medium (DSM) produce una proteína de choque termico llamada GroEL de 60 kDa, la cual induce el aumento de los niveles de anti-inflamatorio IL-10 y pro-inflamatorio IL-12 en THP-1 dendritic cells (Uesugi et al. 2023).

One of the most essential items discussed about the therapeutic use of probiotics in cancer is to guarantee their safety in this sense. In the present work, we demonstrate that the postbiotic *B. mojavensis* has no cytotoxic effect on healthy PBMC cells and that the effect is of the type selective in inducing cytotoxicity in cancer cells.

In this regard, parasporins, proteins produced by *B. thuringiensis* during sporulation, have also been shown to have a selective effect. Borin et al. (2021) reported that parasporins A13-2 and A13-5 decrease the viability of MCF7 breast cancer cells but not on PBMCs and that A13-2 at concentrations of 0.03 to
4.13 µg/mL does not induce hemolysis in erythrocytes. In fact, on the contrary, A13-5 at a concentration of 4 µg/mL increased the viability of PBMCs, did not generate changes in nitric oxide levels, and decreased reactive oxygen species. With all the results, it is shown that the postbiotic produced by the strain of B. mojavensis isolated from Pulque has potential applications in the treatment of CRC, not only due to its effect in reducing cell proliferation but also due to its ability to inhibit migration, counteract the proliferative effect that is induced by LPS and does not induce cytotoxicity in healthy cells. It is essential to continue with future studies in order to identify the metabolites and proteins it produces.

**Declarations**

**Acknowledgements**

We appreciate the technical and administrative support of the laboratories, especially Héctor Galvan Ramos and María Isabel Galvez Soto.

**Author contribution**


**Funding:** This work was partially funded by Mexican Academy of Sciences (AMC), L’Oréal-Mexico, the UNESCO Office in Mexico, the Mexican Commission for Cooperation with UNESCO (CONALMEX), and UPAEP research fund.

**Availability of data and materials:** Please contact author for data request

**Ethics approval and consent to participate.** This article does not contain any studies with human participants or animals performed by any of the authors.

**Consent for publication.** All authors agree to be published.

**Competing interests:** The authors declare that they are clear of any financial or personal conflicts that might have an effect on the research reported in this study.

**References**


**Figures**

**A)**

![Graph showing viability of SW480 cells](image)

**B)**

![Images showing cell morphology](image)

*Figure 1*
Effect of *B. Mojavensis* postbiotic on the viability of SW-480 cells. The cells were incubated with MRS as a control or 25/50 μg/mL of the postbiotic of *B.mojavensis* for 24 h. A) Cell viability was measured by MTT assays. B) Stained with crystal violet. Scale bar = 100 μm. Results are expressed in percentage of control. The data are mean ± SEM (n=3). p < 0.05 were considered statistically significant. **** p ≤ 0.0001 all of them vs. control.

Figure 2

Effect *B. Mojavensis* postbiotic on LPS-induced SW-480 cell viability assessed by MTT assay. Results are expressed in percentage of control. The data are mean ± SEM (n=3). p < 0.05 were considered statistically significant. **** p ≤ 0.0001 all of them vs. control.
Figure 3

Effect of *B. mojavensis* postbiotic on cell migration in SW480. Micrographs at 0 and 24 h of control cells and cells treated with 25 or 50 μg/mL of postbiotic *B. mojavensis* for 24 h. Scale bar = 100 μm.
Figure 4

Effect of *B. mojavensis* postbiotics on PBMCs cell viability assessed by the MTT assay after 24 hours of treatment. Results are expressed in percentage of control. The data are mean ± SEM (n=3). **** p ≤ 0.0001 all of them vs. control.