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ORIGINAL PAPER

Tapioca starch and skim milk support probiotic efficacy of *Lactiplantibacillus plantarum* post-fermentation medium against pathogens and cancer cells

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Abstract

The production of functional foods containing prebiotic ingredients is an area of particular interest and a very promising market with the potential to dominate the food industry. This study aims to explore the potential of starch-based prebiotic tapioca and skim milk, as low-cost and easily accessible food sources and as natural and "clean label" food ingredients on the probiotic activities of *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*). The results show that concomitant use of the modified tapioca starch and skim milk promotes the antibacterial and anti-cancer properties of *L. plantarum* post-fermentation media pointing out how the functionality of probiotic products can be regulated by growth supplements.

Keywords *Lactiplantibacillus plantarum* · Tapioca starch · Postbiotics · *Pseudomonas aeruginosa* · *Klebsiella pneumoniae* · Colon cancer

Introduction

Probiotics, as "bio-friendly agents", are living microorganisms that provide health benefits to the host when applied in sufficient amounts (LeBlanc et al. 2017). Based on their antimicrobial activities, probiotics have been reported to be used for controlling the rate of potentially harmful intestinal microorganisms. The antagonism of probiotics relies on direct inhibition of microbial growth through the production of antimicrobial compounds and/or through competition for adhesions and nutrients (Danilova et al. 2019).

With the increase in antibiotic resistance in pathogenic microorganisms, it has been of great interest to search for alternative agents with antimicrobial activity. Despite the studies revealing the positive indications of probiotic usage, there is a current attempt to use non-viable probiotic-derived biomolecules, namely postbiotics instead of viable probiotic bacteria because of proposed risks related to consumption of the live form of probiotic cells including undesirable side effects, such as systemic infections and excessive immune stimulation. Interfering with the colonization of commensal gut residents, host-induced gene expressions, antibiotic resistance risks, unforeseeable niche-specific actions, the potentiality to cause opportunistic infections, and bacterial translocation to blood or other tissues are also among the theoretical risks of probiotic consumption. Besides, there are not long-term and comprehensive clinical studies examining the effects of long-term probiotic use in patients. There are also many challenges in using live probiotics including the maintenance of viability and purity during the technological production processes which limit the full potential applications of probiotic cells in the pharmaceutical sector and food production. Thus, researchers are getting more interested in the utilization of non-viable bacteria, bacterial compounds, or postbiotics offering similar or even improved health-beneficial biological activities compared to live probiotics (Karaçam and Tunçer 2021; Rad et al. 2021). As a general

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umbrella term, all functional bioactive compounds produced and secreted by bacteria, namely enzymes, peptides, teichoic acids, peptidoglycan-derived muropeptides, polysaccharides, cell surface proteins, and organic acids are included in the postbiotic definition. Current researches about postbiotics, concentrated especially on bacteriocins and short-chain fatty acids (SCFAs), show their health-promoting effects on the host as alternatives for live probiotic cells (Wegh et al. 2019). Furthermore, certain postbiotic products are known for their potential as novel antimicrobial agents to be used as suitable alternatives to antibiotics and/or to combat pathogenic bacteria, as well as to be applied against food spoilage (Rad et al. 2021). Of note, the use of probiotic-derived postbiotic products alone or in combination with conventional antibiotics might be the smartest and cheapest public health strategy to fight against drug-resistant infectious germs.

Lactic acid bacteria (LAB) is among the most commonly used probiotics (LeBlanc et al. 2017). *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum)* (Zheng et al. 2020) is a widely distributed and versatile lactic acid bacterium having a generally recognized as safe (GRAS) status from the US Food and Drug Administration (US FDA) and the qualified presumption of safety status from the European Food Safety Authorities (EFSA). This probiotic bacterium can be found in dairy products, wine, meat, vegetables, silage, and gastrointestinal, vaginal, and urogenital tracts and used in the fermentation of dairy products including cheese, kefir, sauerkraut, fermented meat products, fermented vegetables, and beverages. The health benefits of *L. plantarum* provide for the development of various probiotic formulations and its antibacterial properties have the potential to be used in biopreservation technology for food safety (Behera et al. 2018). Besides, *L. plantarum* is known not only to suppress the growth of pathogenic bacteria but also to inhibit their attachment to intestinal epithelial cells (Golowczyc et al. 2007), and thus, the strains of *L. plantarum* have been suggested for the treatment and prevention of several human diseases characterized by chronic and/or systemic inflammation (Woo et al. 2014). In addition, postbiotics produced by the strains of *L. plantarum* were shown to exhibit selective cytotoxic effects on various cancer cells including colorectal, liver, breast, cervical, and leukemia cancer cells (Chuah et al. 2019), and inhibit the metastasis of colon cancer cells (Yue et al. 2020). This ubiquitous trait highlights the astonishing capabilities of adaptation and metabolic pathway diversities of *L. plantarum* (Seddik et al. 2017). In this study, we aimed to explore the potential of starch-based prebiotic tapioca and skim milk, as low-cost and easily accessible food sources and as natural and "clean label" food ingredients on the probiotic activities of *L. plantarum.*

Resistant starch is defined as the non-digestible fraction of starch that reaches the colon where it acts as a prebiotic. Tapioca starch (TS) is one of the sources of native resistant starch (RS); typically, it has resistant starch type 3 (RS3; retrograded starch). The RS content of tapioca starch is used as a fermentation substrate for lactobacilli (Arshad et al. 2018). Similarly, our group has recently demonstrated that tapioca starch increases the probiotic potential of the oral microbiota LAB members *Streptococcus salivarius* K12 and *S. salivarius* M18 through modulating different cellular processes (Gurbanov et al. 2020). TS can be modified by chemical, physical, and enzymatic processes to extend its industrial applications (Kasote et al. 2018). In the food industry, TS is widely utilized for improving the taste and texture, providing flowability and smoothness, modulating storage properties such as pH and temperature, and giving consistency or stickiness to a large variety of food products including confectionery, yogurt, and noodles (Shigaki 2015). Furthermore, the addition of TS to dairy products can improve some physicochemical properties of the product by giving a creamy texture, whitish color, and a unique flavor to the final food product and decreasing syneresis (Hosseini and Ansari 2019). In the manufacture of dairy products, it is also very common to use skim milk powder, mostly to enrich the milk base (Isleten and Karagul-Yuceer 2006). Since it contains a relatively high concentration of protein in addition to vitamins and minerals, skim milk powder is a rich source of protein for the growth of LAB, and therefore, it is used in the production of probiotic drinks and probiotic yogurts (Pato et al. 2019). Zhang et al. studied the effect of the skim milk supplementation with different carbon sources (glucose, lactose, galactose, and fructose) on the bacterial counts and exopolysaccharide (EPS) production of *Lactobacillus fermentum* F6 and showed that the addition of sugars to the skim milk enhances the growth and EPS biosynthesis of the bacteria (Zhang et al. 2011). Herein, we describe the growth and biological activity amending effects of the enzymatically modified tapioca starch (tapioca modified starch—TMS) alone and in combination with skim milk powder (SM) on the probiotic bacterium *L. plantarum*. The antimicrobial effects of the cell-free post-fermentation media of *L. plantarum* were investigated on *Pseudomonas aeruginosa* and *Klebsiella pneumonia,* gram-negative, opportunistic pathogens that can cause severe nosocomial infections such as bacteremia, pneumonia, urinary tract infections, and soft tissue infections, especially in immunocompromised individuals. Besides, as important food-borne pathogens in milk and milk products, raw vegetables, meat, fish, and street foods, *Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* can cause serious human diseases (Tunçer and Karaçam 2020). In vitro anti-cancer activities of *L. plantarum* post-fermentation media on the colon cancer cells were also investigated in this study to evaluate if the nutrient supplementation affects the competence of the probiotic bacteria.

Functional foods are described to be those wholes, fortified, enriched, or enhanced foods that offer health benefits

beyond the supplying of essential nutrients when they are consumed at efficacious levels as part of a regular diet. As a functional food, RS inclusion into the commercial foods provides nutritional benefits like fiber fortification, functional properties like improved texture, and organoleptic properties such as crispiness in addition to its health benefits, including increased insulin sensitivity, reduced blood sugar levels, and appetite (Gourineni et al. 2020). Therefore, RS can be considered as a part of a healthier and blood-sugar-friendly diet. Both RS and skim milk are used as food ingredients to support the rheological, structural, and sensory properties of a dietary product. It is important to highlight that consumption of functional foods and/or food ingredients with health outcomes requires scientific evidence. Considering that diet can modulate gut microbiota and probiotics are a major component of the emerging functional food market, this study is important to show the modulation in the effectiveness of *L. plantarum* postbiotics through skim milk and RS containing tapioca, as low-cost and easily accessible food sources.

Materials and methods

Preparations of growth media and bacterial growth conditions

To evaluate the effect of the medium ingredients on the growth kinetics of *L. plantarum* and the antibacterial and anti-cancer effects of the *L. plantarum* cell-free supernatant, the probiotic bacteria were incubated with TMS and SM alone or in combination. TMS was dissolved in dH_2O by boiling on a Bunsen burner. Regular De Man, Rogosa, and Sharpe (MRS) broth medium components (peptone from casein 10 g/L, meat extract 8 g/L, yeast extract 4 g/L, dipotassium phosphate 2 g/L, Tween 80 1.1 mL/L, di-ammonium hydrogen citrate 2 g/L, sodium acetate 5 g/L, magnesium sulfate 0.2 g/L, and manganese sulfate 0.04 g/L) except glucose, were added on dissolved TMS and autoclaved for 15 min at 121 °C and cooled at room temperature (RT). As a result of flame-boiling and subsequent autoclaving, the sterile conditions required for the culture process were provided, and also the resistant starch amount of TMS to be metabolized by the probiotic bacteria was increased (Kasote et al. 2018). Skimmed milk powder (0% fat, Sigma) was dissolved in dH_2O and sterilized at 90 °C for 5 min, and added into the sterilized and cooled MRS medium with or without TMS. Otherwise specified, the MRS medium was prepared as glucose-free (G-) to monitor the effect of the TMS solely as a potential primary carbon source. When indicated, glucose (20 g/L) containing MRS medium (G +) was used as a positive control for comparison. *L. plantarum* B-1846 strain (referred to as Lp) was cultured under low oxygen conditions as described before (Gurbanov et al. 2020) at 37 ˚C in a shaking incubator (160 rpm). The media used for *L. plantarum* (Lp) growth are given in Table 1. The w/v concentrations of SM, TMS, and TMS + SM combinations were arranged to 1.0, 0.5, and 0.5 + 1.0% in MRS broth, respectively. The growth medium which has not been inoculated with the probiotic bacteria but incubated under the same conditions was used as "only medium (OM)" control.

Determination of growth dynamics

For the determination of growth dynamics of the bacteria cultures, the optical densities of the cultures at 600 nm (OD_{600}) were measured every 2 h for 72 h. To determine the probiotic viability for each experimental condition, CFUs (colony-forming units) were also counted at different stages of growth and expressed as CFU/mL. For this, 100 µL samples were subjected to serial dilutions (10^{-1}–10^{-7}) in 1 mL MRS broth and 100 µL of each dilution was spread on MRS agar plates. The plates were incubated at 37 °C for 48 h. At the end of the incubation, the colonies were counted and calculated as CFU/mL.

Obtaining the cell-free post-fermentation media

To be used in the biofunctionality studies, cell-free supernatants were obtained as reported previously (Tunçer and Karaçam 2020). Briefly, *L. plantarum* was grown in 15 mL falcon tubes for 14 h as described above and the culture medium was centrifuged at 4 °C, 1844 × *g* for 10 min, after which the supernatants were collected and subjected to microfiltration through 0.22 µm PES membrane. The filtrate, referred to as "SLp" in the manuscript to indicate cell-free supernatant of *L. plantarum*, was aliquoted in 1.5 mL sterile Eppendorf tubes and stored at − 80 °C for further experiments. Where indicated, the pH of the cell-free supernatants and control media were adjusted to pH 7.0 by 1.0 N, sterile NaOH (Tunçer and Karaçam 2020).

Table 1 Experimental conditions: the media used in this study to grow *Lactiplantibacillus plantarum* are given

Lactiplantibacillus plantarum growth media used in this study				
MRS broth G (–)	MRS broth G (–) + 1.0% SM, w/v	MRS broth G (–) + 0.5% TMS, w/v	MRS broth G (–) + 0.5% TMS, w/v + 1.0% SM, w/v	MRS broth G (+)

Lp Lactiplantibacillus plantarum; *TMS* tapioca modified starch, *MRS broth* De Man, Rogosa and Sharpe broth, *SM* skim milk powder, *G* glucose

Determination of antibacterial activity

Antibacterial activities of the cell-free supernatant of *L. plantarum* were analyzed on *Pseudomonas aeruginosa* (ATCC 27,853) and *Klebsiella pneumoniae* (ATCC 700,603) as applied before (Tunçer and Karaçam 2020; Karaçam and Tunçer 2021) with some modifications. For this, *P. aeruginosa* and *K. pneumoniae* strains were grown overnight in tryptic soy broth (TSB) medium, and the day after, the cultures were diluted as 10^6 CFU/mL in TSB followed by 24 h incubation in 96-well plates with 50%, v/v of cell-free post-fermentation media (SLp) and 50%, v/v of growth medium (TSB) in a total volume of 100 µl or only growth media (OM) as control. To determine the pathogen growth inhibitory effects of the probiotic cell-free supernatants, pathogen growth was determined through spectrophotometric measurements at OD_{600} at the end of 24 h incubation. For spot plating assay, *P. aeruginosa* or *K. pneumoniae* was grown in TSB containing 50%, v/v cell-free post-fermentation media (or control growth media-OM) for 24 h were subjected to serial dilutions in TSB and 3.0 µl of each dilution was spotted on agar plates. The plates were incubated overnight at 37 °C before being photographed using the G: BOX Chemi XRQ gel documentation system (Syngene, England).

Cell culture, treatments, and MTT assay

The anti-cancer effects of the probiotic cell-free supernatants were investigated in vitro on HCT-116 colon cancer cells. The cells, a kind gift of Prof. Dr. Sreeparna Banerjee (Middle East Technical University, Ankara, Turkey), were grown and sub-cultured as described previously (Tunçer et al. 2019). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine the effect of the cell-free post-fermentation medium on the colon cancer cells through analyzing the metabolic reduction levels of MTT (Onat et al. 2019). Briefly, HCT-116 cells were seeded as 1×10^4 cells/well of a 96-well plate and the day after, treated with RPMI-1640 complete growth medium containing 30%, v/v post-fermentation cell-free supernatant (SLp) or 30%, v/v control medium (OM). Following 24 h of incubation in a 5% CO_2, 95% air incubator at 37 °C, the media were discarded and the cells were incubated with 100 µL of RPMI-1640 culture medium containing 1.2 mM of MTT for 4 h at 37 °C. 100 µL of the SDS-HCl solution (1 g of SDS in 10 mL of 0.01 M HCl) was used to dissolve the MTT reduction product, formazan crystals, by incubating the plates at 37 °C for 16 h. The intensity of the product color was measured at 570 nm using a microplate reader (Thermo Fisher Scientific) and MTT reduction was expressed as a percentage relative to the control medium (OM) treated cells. Absorbance values at 570 nm obtained from the wells containing complete medium with MTT and SDS-HCl but without cells were used as blanks.

Western blot

To evaluate if treatments of HCT-116 colon cancer cells with post-fermentation media of *L. plantarum* trigger apoptosis, western blot was used to determine the changes in the expression levels of the anti-apoptotic protein X-linked inhibitor of apoptosis (XIAP). For this, HCT-116 cells were seeded as 3×10^5 cells/well in a 24-well plate. Followed by overnight incubation, the cells were treated with 30%, v/v post-fermentation cell-free supernatant (SLp) or control medium (OM) for 24 h at 37 °C in a 5% CO_2, 95% air incubator. At the end of incubation, the medium was discarded and total cell proteins were isolated as described before (Tunçer et al. 2020). Briefly, the wells were washed with ice-cold phosphate-buffered saline (PBS) and 60 µL of T-PER Protein Extraction Reagent (Thermo Fisher Scientific) containing protease inhibitor cocktail (Roche, Switzerland) and phosphatase inhibitor (Roche) was used for the lysis of the cells using cell-scraper. The lysates were collected in Eppendorf tubes and incubated on ice for 30 min by vortexing vigorously every 10 min. Proteins were collected by 15 min centrifugation which was carried out at 4 °C, $14.000 \times g$. Pierce™ Coomassie Plus (Thermo Fisher Scientific) reagent was used to determine protein concentrations by measuring the absorbance values at 595 nm and transforming the values into concentrations according to a BSA standard curve.

For western blot, 15 µg of proteins was separated by 10% SDS-PAGE after denaturing the samples by boiling at 95 °C for 6 min in a 6X loading dye composed of 375 mM Tris–HCl pH 6.8, 12% SDS, 30% β-mercaptoethanol, 30% glycerol, and 0.02% bromophenol blue. The proteins were then transferred to polyvinylidene difluoride (PVDF) membrane at 200 mA for 1.5 h using Hoefer TE70XP semi-dry blotting system (Massachusetts, USA). Followed by 1 h of blocking in 5% skim milk powder in Tris-buffered saline containing 0.1% Tween 20 (TBS-T), the membrane was immunoblotted overnight at 4 °C with XIAP antibody (cat. no: sc-11426, Santa Cruz Biotechnology, Texas, USA; 1:300 dilution). Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH, cat. no: sc-25778, Santa Cruz Biotechnology; 1:1000 dilution) was used as a loading control. The bands were visualized using Clarity ECL Substrate (Bio-Rad, California, USA) in a G: BOX imaging system, equipped with GeneSys image capture software (Syngene) after 1 h incubation at RT with anti-rabbit antibody (cat. no: bs-0295G-HRP anti-rabbit, Bioss, Massachusetts, USA; 1:1000 dilution) conjugated with horseradish peroxidase.

Before carrying out the western blot, the cells were observed by Nikon Eclipse TS100 (Japan) inverted light

microscope with 4X objective lens and photographed with Toupcam HD camera (China).

Statistical analysis

GraphPad Prism 6.01 (GraphPad, USA) was used for the statistical analysis and plotting of the graphs. The experiments were repeated at least three times with at least four technical replicates and the results were expressed as means ± standard error of the mean (SEM). The data were analyzed with a t test and the degrees of significance were denoted as $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, and $****P \leq 0.0001$.

Results

Tapioca starch and skim milk change the growth dynamics of *L. plantarum*

Over 72 h of incubation, the growth of *L. plantarum* in MRS broth without glucose but with SM (Lp-SM) and TMS (Lp-TMS) alone or as a combination (Lp-TMS + SM) was monitored by measuring the optical densities of the cultures at 600 nm (Fig. 1a). Throughout the incubation, changes in the pH of the growth medium were also determined by measuring the pH of the bacterial cultures for every 2 h (Fig. 1b). Since it is well known that certain bacteria can attach the starch granules and this attachment ability results in a decrease in the optical density of the population (Minato and Suto 1979), we also investigated the growth pattern of the *L. plantarum* by determining CFU counts (Table 2). Following the previous reports (Minato and Suto 1979; Crittenden et al. 2001), inconsistency between the optical densities and CFU counts indicates the starch adhesion of the probiotic bacteria. Therefore, CFU counts reveal that supplementation of the glucose-free growth medium with SM enhances the *L. plantarum* growth and the addition of the tapioca starch to the skim milk powder containing medium (TMS + SM) further encourages the growth of the probiotic bacteria. In addition, as an indicator of bacterial growth, a drop in the pH of the fermentation medium, which is caused by the production of organic acids, mainly lactic acid (Ummadi and Curic-Bawden 2010), was also observed for *L. plantarum* treated with SM and/or TMS + SM: both Lp and Lp-TMS groups showed a similar drop (pH 5.5) before reaching the stationary phase whereas the pH levels of the Lp-SM and Lp-TMS + SM groups were dropped to around 5.0.

Based on the growth curves, the initiation of stationary phases was determined at around 14 h. Since the concentration of the antimicrobial substances of *L. plantarum* was reported to reach a maximum at the onset of the stationary phase of the growth (Messi et al. 2001), the probiotic supernatants were collected after 14 h of incubation to be used in the biofunctionality experiments.

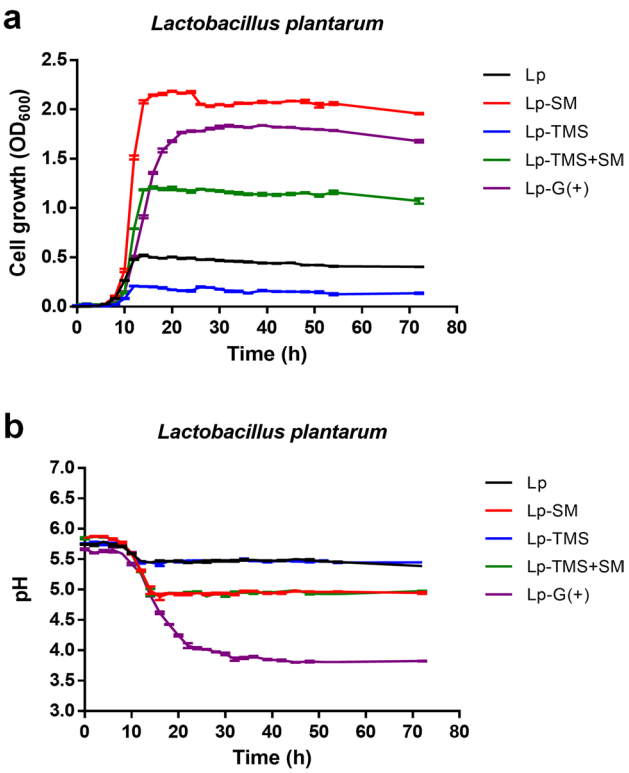

Fig. 1 Growth dynamics of *Lactiplantibacillus plantarum* grown in the presence of tapioca modified starch and skim milk. Over 72 h of incubation, **a** growth of *L. plantarum* was followed by determining optical densities at 600 nm and **b** pH of the cultures was measured for every 2 h. The growth and pH curves were plotted as a function of time. The results belong to three independent experiments. The data were presented as means ± SEM. *Lp L. plantarum, SM* skim milk, *TMS* tapioca modified starch, *G (+)* the presence of glucose

L. plantarum cell-free post-fermentation media inhibit the growth of the pathogens *Pseudomonas aeruginosa* and *Klebsiella pneumonia*

Post-fermentation cell-free supernatants of *L. plantarum* (SLp) collected from different experimental groups, namely SLp, SLp-SM, SLp-TMS, and SLp-TMS + SM were applied as 50% v/v (in TSB medium) to test their inhibitory activities against the pathogens *P. aeruginosa* (Fig. 2) and *K. pneumoniae* (Fig. 3). SLp-G (+) was used as positive control and the effect of the OM of each experimental condition was also investigated. After 24 h of incubation, the inhibitory activities of the probiotic supernatants were analyzed by measuring the optical densities of the pathogen cultures at 600 nm and through spot plate assay. For both *P. aeruginosa* and *K. pneumonia,* all experimental groups of *L. plantarum* diminished the cell viability of the pathogens; however, the most prominent inhibitions were obtained when the pathogens

Table 2 The colony-forming units (CFUs) of *Lactiplantibacillus plantarum* grown in the presence of skim milk and tapioca starch

Experimental groups	CFU/mL			
	10 h	14 h	34 h	72 h
Lp	$8.833\times10^8\pm1.764\times10^6$	$2.557\times10^8\pm1.417\times10^7$	$1.850\times10^8\pm1.732\times10^6$	$9.400\times10^8\pm3.055\times10^6$
Lp-SM	$1.747\times10^8\pm1.525\times10^7$ (**)	$1.043\times10^9\pm1.202\times10^7$ (****)	$1.337\times10^9\pm4.485\times10^7$ (****)	$1.000\times10^7\pm0.0$ (***)
Lp-TMS	$8.967\times10^8\pm1.041\times10^7$ (ns)	$2.745\times10^8\pm4.550\times10^7$ (ns)	$2.677\times10^8\pm3.528\times10^6$ (****)	$1.807\times10^8\pm1.719\times10^7$ (**)
Lp-TMS + SM	$1.565\times10^8\pm2.500\times10^6$ (***)	$2.235\times10^9\pm1.350\times10^8$ (***)	$6.850\times10^8\pm5.500\times10^7$ (**)	$2.000\times10^7\pm0.0$ (***)

For each time point, viable cell numbers are expressed as CFU/mL. The results, belonging to three independent replicates, are given as mean ± SEM. The statistical significances were analyzed using a *t* test with respect to the Lp groups.

Lp Lactiplantibacillus plantarum grown in glucose-free MRS, *Lp-TMS L. plantarum* grown in glucose-free MRS containing tapioca modified starch, *Lp-SM L. plantarum* grown in glucose-free MRS containing skim milk powder, *Lp-TMS + SM L. plantarum* grown in glucose-free MRS containing skim milk powder and tapioca modified starch

*stands for the level of significance with Lp

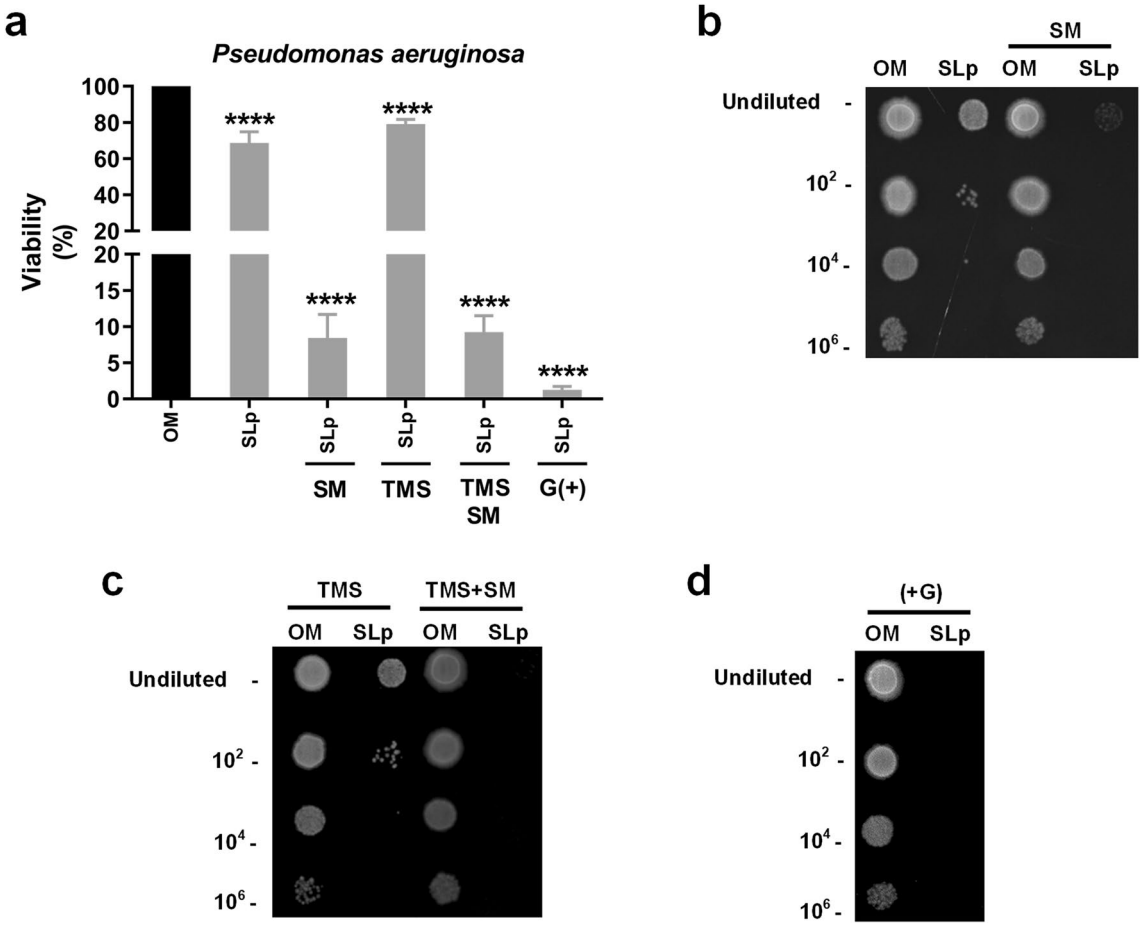

Fig. 2 The cell-free post-fermentation medium of *Lactiplantibacillus plantarum* hinders the vegetation of *Pseudomonas aeruginosa.* **a** The percent viability of *P. aeruginosa* incubated for 24 h with 50% (v/v) *L. plantarum* post-fermentation cell-free media or corresponding "only media (OM)" as controls. **b–d** Spot plate assays were used for visualizing cell growth followed by the incubation with the post-fermentation cell-free media or with the control media. The results were presented as mean ± SEM. *t* test was used to compare the SLp, SLp-SM, SLp-TMS, SLp-TMS + SM, and SLp-G (+) groups with the corresponding OM groups. *SLp* supernatant of *L. plantarum*, *SLp-SM* supernatant of *L. plantarum* grown in the presence of skim milk, *SLp-TMS* supernatant of *L. plantarum* grown in the presence of tapioca modified starch, *SLp-G (+)* supernatant of *L. plantarum* grown in the presence of glucose

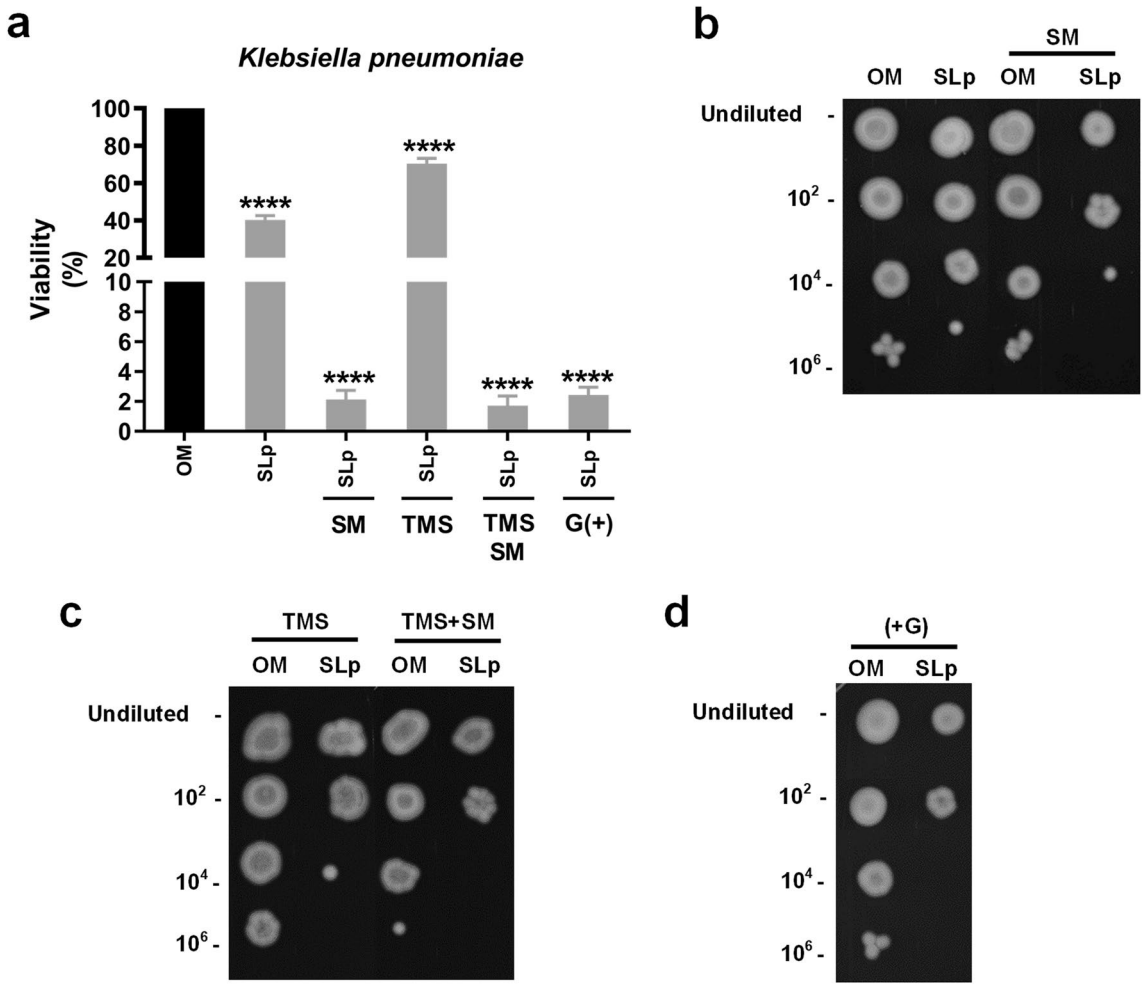

Fig. 3 The cell-free post-fermentation medium of *Lactiplantibacillus plantarum* hinders the vegetation of *Klebsiella pneumoniae*. **a** The percent viability of *K. pneumoniae* grown with 50% (v/v) *L. plantarum* post-fermentative media or corresponding "only media (OM)" as controls. **b–d** Spot plate assay demonstrating the growth of *K. pneumonia* after 24 h incubation with the post-fermentation media or control media. The results were presented as mean ± SEM. *t* test was used to compare the SLp, SLp-SM, SLp-TMS, SLp-TMS + SM, and SLp-G (+) groups with the corresponding OM groups. *SLp* supernatant of *L. plantarum*, *SLp-SM* supernatant of *L. plantarum* grown in the presence of skim milk, *SLp-TMS* supernatant of *L. plantarum* grown in the presence of tapioca modified starch, *SLp-G (+)* supernatant of *L. plantarum* grown in the presence of glucose

had been incubated with the supernatants collected from *L. plantarum* treated with SM alone and in combination with TMS. Of note, the post-fermentation medium collected from SM or TMS + SM incubated *L. plantarum* showed a drastic growth inhibitory activity on both *P. aeruginosa and K. pneumonia,* and the degree of the inhibition was similar to the glucose treated (G +) counterpart which was used as a positive control in this study.

The pH of the MRS medium is around 5.7. As presented in Fig. 1, the growth of *L. plantarum* reduces the pH of the medium near 5.0 in the presence of SM alone or together with TMS (TMS + SM). On the other hand, the presence of glucose in the growth medium reduces the pH of the medium to around 4.0. Thus, we asked whether these changes in pH can affect the growth of *P. aeruginosa* and *K. pneumonia*. For this purpose, the pH of the cell-free supernatants (and corresponding control media) was adjusted to pH 7.0, the pH of the TSB medium, and the pathogens were treated with the probiotic fermentation media (50% v/v in TSB medium) as described above. As shown in Supplementary Fig. 1, although the fermentation media still show inhibitory effects on the pathogen bacteria, the decreased antimicrobial activity at pH 7.0 suggests that the fermentation media require a slightly acidic environment to show the anti-pathogenic activity. LAB produces acidic end products which create unfavorable conditions for the growth of many pathogenic bacteria by reducing the pH of the environment (Barbour et al. 2020). The undissociated form of the organic acids dissociates in the cytoplasm and reduces the intracellular pH or the intracellular accumulation of the ionized form of

the organic acid can lead to the death of the pathogen (Bermudez-Brito et al. 2012). Furthermore, these results suggest that in a slightly acidic environment, the anti-pathogenic activity of *L. plantarum* postbiotics was potentially caused by the combined effects of the postbiotics which can be proteinaceous in nature and more active at acidic pH (Karaçam and Tunçer 2021), and the organic acids produced by *L. plantarum* in which the activities have been lost by neutralizing the environmental pH (Tunçer and Karaçam 2020).

L. plantarum cell-free post-fermentation media show an anti-cancer effect on colon cancer cells in vitro

To evaluate if skim milk and tapioca starch as medium supplements affect the anti-cancer activity of *L. plantarum,* HCT-116 colon cancer cells were treated for 24 h with the post-fermentation cell-free culture medium collected from *L. plantarum* incubated with skim milk (SLp-SM), tapioca modified starch (SLp-TMS), or together with skim milk and tapioca modified starch (SLp-TMS + SM) in the MRS medium without glucose. MRS medium containing glucose (SLp-G +) was used as a positive control, as previously described, and the post-fermentation cell-free supernatant of *L. plantarum* incubated in glucose-free MRS medium (SLp) was used for comparison (Yılmaz and Şimşek 2020). For quantitative analysis, an MTT assay was applied to measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity at the end of the treatment (Karaçam and Tunçer 2021). As shown in Fig. 4a, treatment with the cell-free supernatant *L. plantarum* grown in MRS medium containing glucose (SLp-G +) exerted a drastic growth inhibitory effect on HCT-116 colon cancer cells, in accordance with the previous report (Chuah et al. 2019). A very similar inhibitory activity was also obtained with the cell-free supernatant collected from the probiotic bacteria incubated with SM and TMS together (SLp-TMS + SM). The cells incubated with the above-mentioned cell-free supernatant groups were also visualized under a light microscope and photographed. The detrimental effects of the probiotic supernatants, especially SLp-TMS + SM, on HCT-116 cells are shown in Fig. 4b: the cells are detached, shriveled, grainy and dark-colored in appearance.

The apoptosis-inducing activity of the probiotic supernatant collected from *L. plantarum* incubated with SM alone or in combination with TMS was also investigated. As shown in Fig. 4c, the cell-free supernatants obtained from *L. plantarum* incubated in the presence of SM, TMS, or combination (SM + TMS) decreased the expression of the anti-apoptotic protein XIAP, indicating that the supernatants were capable of inducing apoptosis. Autophagy is a physiological process of degradation and recycling of organelles, long-lived proteins, and other intracellular components and is considered as a cell survival mechanism as a response to stresses such as nutrient deprivation, whereby cellular materials are enclosed in double-membrane layered organelles known as autophagosomes for degradation (Tunçer and Banerjee 2017). XIAP was shown to function both in apoptosis and autophagy. Its expression is elevated in different cancers, including colon cancer. It is believed that the anti-apoptotic ability of XIAP is primarily related to the direct binding and inhibiting of caspases, the apoptotic proteases that are responsible for the initiation and execution of apoptosis. Following a range of apoptotic stimuli, XIAP is phosphorylated by Akt and this phosphorylation leads to the stabilization of XIAP. Stabilized XIAP promotes XIAP-dependent proteasomal degradation of caspases 3, 7, and 9. The result is a block of apoptosis and tumor chemoresistance. It can be suggested that this is the situation for the cells treated with the post-fermentation medium obtained from SM, TMS and SM + TMS incubated probiotics. Starvation, on the other hand, inhibits Akt and unphosphorylated XIAP undergoes autoubiquitylation and degradation via the proteasome. Subsequently, Mdm2 is stabilized and induces p53 degradation, releasing the block on autophagy. In another word, under starvation, loss of XIAP (via degradation) leads to autophagy (Merlo and Cecconi 2013). Therefore, decreased expression of XIAP which was seen both in OM (glucose-free) and SLp (glucose-free) treated cells may be caused by nutrient deprivation (i.e., without glucose). Finally, it is well known that increased autophagy in nutrient-deprived or growth factor-withdrawn cells allows cell survival by inhibiting apoptosis (Thorburn 2008). Thus, we think that autophagy may occur in the cells treated with MRS, without glucose (both for OM control and SLp).

Discussion

Gut microbes, mostly bacteria, have different functions and effects on health through modulating the physiological functions of the host such as digestion, metabolism, and immune responses (LeBlanc et al. 2017). Therefore, intestinal flora can have beneficial or detrimental effects on an individual's health status, depending on the colonization of commensal or harmful bacteria, respectively. When the balance between the "beneficial" and "harmful" bacteria in the flora changes, a pathological process called "microbial dysbiosis" can occur. Recent studies have shown that an abnormal or altered gut microbiome (dysbiosis) is associated with a variety of diseases. Since dietary habits can affect the gut microbial diversity, dietary intervention of a prebiotic and/or probiotic has been gaining attention to modulate the composition and metabolic activity of the gut microbiota (Matijašić et al. 2016). Prebiotics are food components that are usually indigestible carbohydrates that can selectively stimulate the

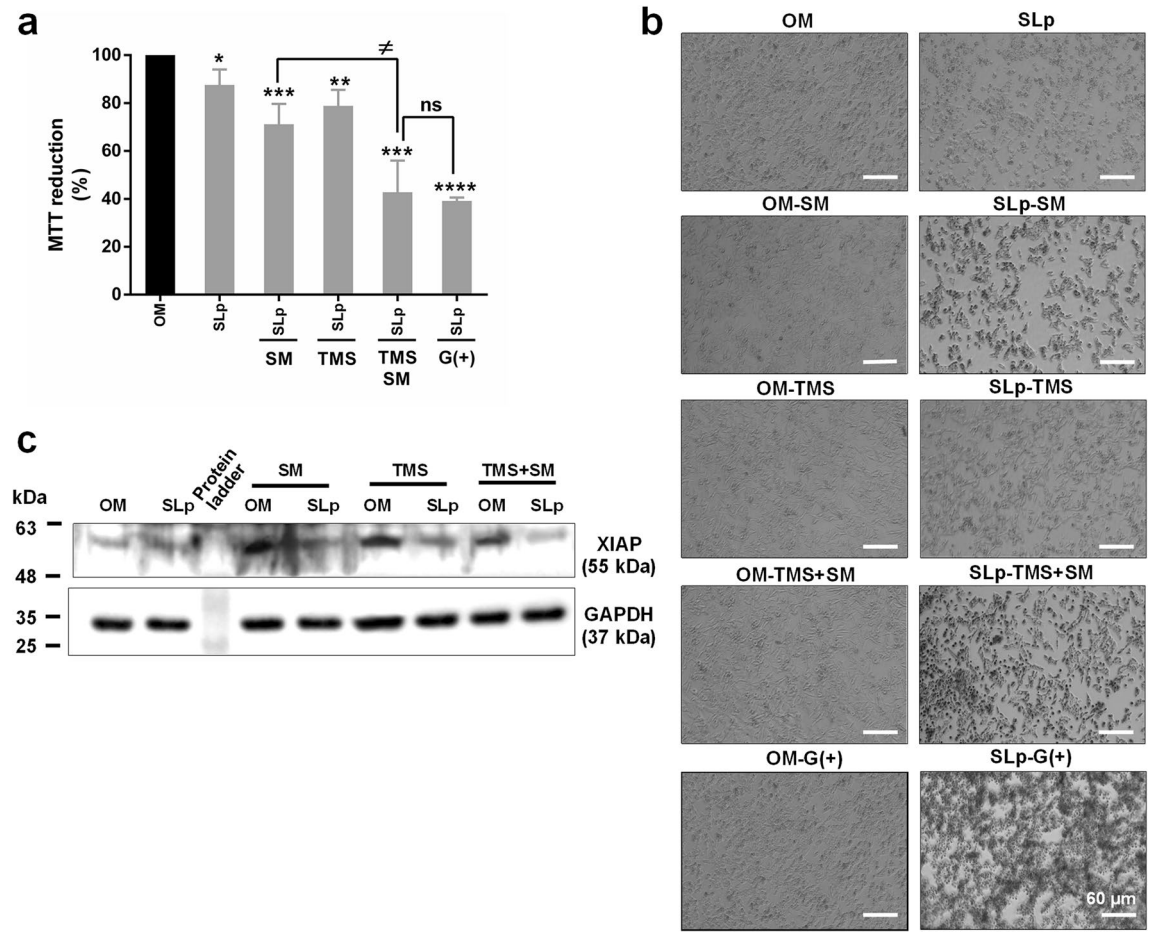

Fig. 4 The anti-cancer potential of *Lactiplantibacillus plantarum* is affected by growth medium supplements in vitro. HCT-116 colon cancer cells were treated for 24 h with the post-fermentation cell-free culture medium collected from *L. plantarum* incubated with skim milk (SLp-SM), tapioca modified starch (SLp-TMS), or together with skim milk and tapioca modified starch (SLp-TMS + SM) in the MRS medium without glucose. **a** MTT reduction levels, as an assessment of metabolic viability, show the growth inhibitory activities of the post-fermentation media. For each treatment group, the reduced levels of MTT were normalized with respect to control medium (OM) treated cells and given as percent (%) change. The probiotic cell-free medium collected from *L. plantarum* incubated with the MRS medium without (SLp) or with glucose (SLp-G+) was used for comparisons. The experiments were repeated two times each with six technical replicates and represented as mean ± SEM. A *t* test was used for statistical analysis. "*" stands for the level of significance with "OM" and "≠" was used to show the statistical significance between SLp-SM and SLp-TMS + SM groups. **b** At the end of the treatments, the microscope images of HCT-116 cells were obtained with an inverted light microscope with a 4X objective lens. **c** Western blot image shows the expression levels of the anti-apoptotic protein XIAP in HCT-116 cell incubated with control medium (OM) or *L. plantarum* post-fermentation cell-free culture medium. GAPDH was used as a loading control. *SLp* supernatant of *L. plantarum*, *SLp-SM* supernatant of *L. plantarum* grown in the presence of skim milk, *SLp-TMS* supernatant of *L. plantarum* grown in the presence of tapioca modified starch; *SLp-G (+)* supernatant of *L. plantarum* grown in the presence of glucose

growth and metabolic activity of probiotic bacteria such as *Lactobacillus* species (LeBlanc et al. 2017). In this study, we aimed to investigate the enzymatically modified, commercially available food-grade tapioca starch (TMS) on the growth kinetics, anti-pathogenic and anti-cancer activities of the probiotic *L. plantarum* which can colonize the intestinal tract of humans with a proven capability to survive gastric transit (Le and Yang 2018).

In the context of dietary interventions with prebiotics, the consumption of dietary fibers, as natural sources of RS, is an area of particular interest. RS is attributed to dietary starch and starch degradation products that cannot be digested in the small intestine but fermented in the large intestine conferring benefits to human health. RS3 also referred to as retrograded starch or crystalline non-granular starch collects particular interest in the food industry among different RS types because it can maintain its nutritional functionality and thermal stability during cooking (Gurbanov and Tunçer 2021). Having a lower caloric value and unique functional properties and physiological benefits, RS is categorized as a "functional food" ingredient. Several potential health benefits attributed to RS consumption such as slowing digestion,

reducing abdominal fat and cholesterol, increasing systemic insulin sensitivity, and positive effects on large bowel health along with anti-cancer properties (Wang et al. 2019; Gurbanov and Tunçer 2021). Functioning as a prebiotic, RS has been also reported to encourage the growth of probiotic bacterial species and to confer benefits to gut health, particularly in the large intestine where RS is fermented. By decreasing the concentration of secondary bile acids, ammonia and phenol content, enhancing the production of SCFAs, promoting the absorption of zinc, calcium, and magnesium ions, and reducing the intestinal pH, RS can provide a range of physiological benefits to the host as well as digestive comfort (Gurbanov and Tunçer 2021). As noted, TS is one of the sources of native resistant starch, and here we show that the combination of tapioca modified starch (TMS) with skim milk (SM) enhances the viable bacterial counts, and therefore, it can be utilized to support the growth of probiotic *L. plantarum*.

LAB is one of the potential natural sources of antimicrobial agents due to the variety and quantities of metabolites present in the cell-free post-fermentation medium (Wang et al. 2020). Previous studies report that LAB-derived fermentation media exert inhibitory functions against many human pathogens such as *Listeria monocytogenes, Staphylococcus aureus, Staphylococcus epidermis, Bacillus subtilis, Salmonella typhimurium, Escherichia coli* O157: H7*, Salmonella typhimurium, Enterobacter cloacae, Enterococcus faecalis, Clostridium difficile, Helicobacter pylori, Shigella sonnei Staphylococcus aureus Campylobacter jejuni, Candida albicans, P. aeruginosa*, and *K. pneumoniae* (Tunçer and Karaçam 2020). Our results show that post-fermentation media of *L. plantarum* (SLp) can efficiently inhibit the growth of *P. aeruginosa* and *K. pneumoniae*; however, supplementation of the growth medium with SM and/or TMS + SM increases the pathogen growth inhibitory activity of the post-fermentation media further. Inhibition of pathogenic bacteria has been attributed to postbiotics produced and released by LAB into the fermentation media (Yazgan et al. 2021) and productions of the postbiotics have been reported to be greatly affected by the composition of the growth medium (Nataraj et al. 2020). Thus, it can be suggested that SM and/or TMS + SM supplementation can change the composition of the postbiotic metabolites and, therefore, modulate the growth inhibitory efficacy of the post-fermentation medium on the pathogenic bacteria. *P. aeruginosa* and *K. pneumoniae* are opportunistic pathogens, and treatment of infections caused by these pathogens is extremely difficult because of their resistance to multiple antibiotics (Elgendy et al. 2018). As alternatives to antibiotics, the main antimicrobial actions of postbiotics rely on the acidification of the cellular cytoplasm, prevention of energy production and regulation, as well as suppression of the pathogenic growth through driving oxidation of cellular components and triggering pore formation in the target cell membranes (Rad et al. 2021). The presence of the outer membrane in Gram-negative bacteria creates a physical barrier to resist the passage and binding of antimicrobials produced by LAB (Pehrson et al. 2015). However, outer membrane destabilization can sensitize bacteria to bacteriocin attack: binding of LAB-produced lactic acid can liberate the lipopolysaccharides (LPS) from the outer membrane of the target and thereby act as an osmotic agent to disrupt the bacterial membrane (Alakomi et al. 2000). Bacteriocins can increase the damage further by inducing morphological and functional changes of the cellular components such as proteins and, therefore, inhibit the activity of the intracellular biomolecules after passing through the target's damaged outer membrane (Helander et al. 2001). Bacteriocins produced by the *L. plantarum* are called plantaricins and both *L. plantarum* and plantaricins have been getting great interests in different areas as food biopreservatives and/or starters in food production including dairy products and as therapeutic agents for treating pathogenic bacteria-caused diseases and conditions, such as irritable bowel syndrome and chronic infections (Abdulhussain Kareem and Razavi 2020). Since medium composition is very well known to affect the production of plantaricins (Abdulhussain Kareem and Razavi 2020), one can suggest that the presence of SM and TMS in the growth medium change the amounts, composition, and/or biomolecular diversity of the plantaricins along with the other postbiotic products.

In addition to anti-pathogenic properties, cell-free supernatant of *L. plantarum* was also reported to have cytotoxic effects on cancer cells (Chuah et al. 2019). Apoptosis induction in colon cancer cells through treatment with cell-free supernatant collected from the different *L. plantarum* strains grown in glucose-containing MRS medium have been evaluated before (Nami et al. 2014; An and Ha 2016). Our results show that the fermentation medium obtained from *L. plantarum* grown in the presence of both SM and TMS exhibited an enhanced cytotoxic activity on colon cancer cells compared to the cell-free supernatant obtained from the probiotic bacteria incubated with SM or TMS alone, although when used separately, both additives also reduced XIAP expression as an indication for apoptosis promotion. Similar to the results obtained from the anti-pathogen activity analysis, these results also emphasize that anti-cancer activity of *L. plantarum* postbiotics can be altered by modifying the growth conditions of the probiotic.

RS offers health benefits to consumers, especially for diabetics with overweight or obese, such as improving fasting glucose, fasting insulin, insulin resistance, and sensitivity (Wang et al. 2019). It can be also used as a stabilizer during storage or emulsifier to elicit creamier and denser sensations as a natural and "clean label" food ingredient (Lobato-Calleros et al. 2014; Morell et al. 2015). It has been shown that

the addition of RS to the reduced-fat yogurts prepared with skim milk powder reduces syneresis by producing large-size casein flocs (Lobato-Calleros et al. 2014) in accordance with the previous reports suggesting that starch granules in skim milk gels can increase the milk protein concentration during swelling by absorbing water (Zuo et al. 2008; Singh and Byars 2009). Thus, besides enhancing the rheological, structural, and sensory properties of dairy products, tapioca starch and skim milk can be applied as a manufacturing strategy in the production of functional foods and probiotic dairy products to offer health benefits to the consumers besides offering a prolonged shelf life to dairy products.

Since the compositional and functional microbiome changes have been reported to be associated with the pathogenesis of common multifactorial diseases (Fan and Pedersen 2021), it can be proposed that altered metabolite profiles of the microbiome may have an impact on the physiological functions of the host, such as immune responses and metabolism (Blacher et al. 2017). Furthermore, recent data underlies the microbiota modulating effect of diet and suggest that dietary strategies affect the physiology and health of the host through manipulating microbial composition, diversity, activity, and stability (Wang et al. 2019). In this regard, further studies are needed to describe the functional and biological consequences of the interactions between the various prebiotics with probiotic gut bacteria (Gurbanov et al. 2020). While analyzing the interaction between diet and microbiome, expanding this current issue on the axis of postbiotic products will add value to scientific knowledge, undoubtedly. Current literature shows that postbiotics can be applied as promising tools in food practice to prevent microbial corruption and develop functional foods. The findings of this study point out that concomitant use of skim milk and tapioca starch promotes the antibacterial and anti-cancer properties of the probiotic *L. plantarum*. Further studies are aimed to describe the functional probiotic products in which the productions are regulated by the growth supplements. On the other hand, to validate the synbiotic's pre-and probiotic effects, carrying out in vivo trials for the development of new functional formulations is also needed.

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Author contributions HK and SK (graduate students under the supervision of ST and RG) carried out the experiments to determine the probiotic's growth kinetics and the assays for antimicrobial activities. ST carried out experiments on the anti-cancer effects of the probiotic supernatants. RG designated the study and ST and RG analyzed the results and wrote the manuscript. This study is a part of the Master of Science thesis of HK. All the authors contributed to the conception and design of the study and the final manuscript has been read and approved by all the authors.

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Availability of data and materials All data generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability There is not any custom computer code or algorithm used to generate the results reported in the manuscript.

Declarations

Competing interests The authors have declared that no competing interests exist.

Ethics approval Ethics approval is not required in this study.

Consent to participate For this type of study, consent is not required.

Consent for publication Consent for publication is not required in this study.

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