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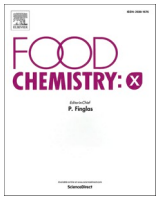

Lactiplantibacillus plantarum MIUG BL21 paraprobiotics: Evidences on inactivation kinetics and their potential as cytocompatible and antitumor alternatives

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ABSTRACT

Two new -biotics concepts, such as paraprobiotics and postbiotics were introduced, with beneficial effects beyond the viability of probiotic. In this study, the effect of individual (thermal, ohmic heating, high pressure, and ultrasound) and combined (ohmic, high pressure and ultrasound in combination with heating) treatments on the inactivation kinetics of *Lactiplantibacillus plantarum* was investigated. Different inactivation rates were obtained, up to 8.18 after 10 min at 90 °C, 2.07 after 15 min at a voltage gradient of 20 V/cm, 6.62 after 10 min at 600 MPa and 3.6 after ultrasound treatment for 10 min at 100 % amplitude. The experimental data were fitted to Weibullian model proposed by Peleg, allowing to estimate the inactivation rate coefficient (*b*) and the shape of the inactivation curves (*n*). At lower concentration, the samples showed both cytocompatibility and antiproliferative effect, stimulating the cell proliferation on both murine fibroblast and human colorectal adenocarcinoma cell lines.

1. Introduction

In the last decades, the popularity and, consequently, the consumption of probiotic bacteria either in fermented foods or as supplements has increased significantly, due to the related health benefits (Macharia et al., 2023). The health benefits of probiotics are provided due to interactions between the gastrointestinal microbiota and the immune system (Adams, 2010), when administered in adequate amounts (Hill et al., 2014). Therefore, it has been demonstrated that probiotic strains are essential in sustaining the equilibrium between the number of other members of the native gut flora and their enzymatic activities (Górska et al., 2019). However, fundamental limitations should be considered when using probiotics in food or/and nutraceuticals. These limitations are related with the ability of probiotics cells to remain viable in the food before consumption. When ingested, the cells should survive in the upper gastrointestinal tract, to live and grow in the tract, while no toxic or pathogenic activity is accepted (Kwok, 2014). Wide ranges of foods containing probiotics are currently available on the market, but from a technological perspective, ensuring cells survival in food matrices or addition of probiotics during processing is still challenging. de Almada et al. (2016) suggested the main technological challenges, such as the survival of probiotics during shelf life and low thermal resistance, that requires the addition of probiotics to food matrices after thermal processing. Therefore, to respond to these complex set of challenges, recent studies have showed that inactivated probiotic cells, known as paraprobiotics, could also exert biological activity for the host (Taverniti & Guglielmetti, 2011; Aguilar-Toalá et al., 2018). These concepts are used to indicate bioactive compounds, which do not fit the traditional definitions of probiotics, prebiotics and/or symbiotics. Therefore, paraprobiotics also called "inactivated probiotics" or "ghost probiotics" were defined by Barros et al. (2020) as "non-viable microbial cells (intact or ruptured) or raw cellular extracts (with complex chemical composition), which when administered (orally or topically) in adequate amounts, confer a benefit to the human or animal consumer". Many health-related benefits of paraprobiotics are reported, such as reducing the risk of opportunistic infections in humans (fungemia, bacteremia, endocarditis

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and sepsis), effectiveness in controlling the postprandial glycemia in healthy adults (Barros et al., 2021). Some other authors suggested the ability to reduce inflammatory responses to vaccine or allergens (fever, arthritis) and suppressing lipid accumulation during adipogenesis (Kim et al., 2021). Moreover, the paraprobiotics may be involved in degradation of mucin and production of deconjugated bile salts and D-lactate, lack of horizontal transference of antibiotic resistance genes to other commensal or pathogenic bacteria in the gut; microbial translocation.

When considering the addition in food, Barros et al. (2020) highlighted the main advantages of using paraprobiotics in food and supplements, such as a simpler and more convenient industrial handling and marketing, significantly limited interaction potential with the food matrix components, allowing shelf-life increase. Additionally, the paraprobiotics are stable in a wide range of pH and temperature, which allows addition to highly acidic food and before thermal processing, thus reducing the risk of post-process contamination. Furthermore, the use of paraprobiotics could contribute to the energy savings, as the products do not require cold chains, while the sensory properties of food are not influenced, thus guaranteeing the consumer acceptability (Barros et al., 2020). Moreover, the inactive cells reduce the risks of bacterial translocation from the gastrointestinal environment to the blood in subjects with a vulnerable immune system (Taverniti & Guglielmetti, 2011), whereas a greater absorption is expected, due to a direct contact with mucus layers and epithelial cells (Piqué, Berlanga, & Miñana-Galbis, 2019).

Different techniques are used to inactivate cells, such as physical methods (heating, gamma or ultraviolet radiation, high hydrostatic pressure, ultrasonication and freeze-drying) or chemical (acid deactivation). However, selection of the appropriate method will depend on the strain and the expected clinical benefit, since each method releases different components with various metabolic functions (Taverniti & Guglielmetti, 2011; de Almada et al. (2016).

To date, there is limited research on the inactivation patterns of different types of treatments on certain bacterial strains with the aim to produce paraprobiotics, whereas analyzing the cytocompatibility and antiproliferative effects of inactive cells is lacking. One of the main challenges in producing paraprobiotics by inactivation processes remain the precise prediction of probiotic cells inactivation rate, without compromising the probiotic benefits. Given the above-mentioned key elements, the aim of this study is to investigate the inactivation patterns of *Lpb. plantarum* MIUG BL21 strain using different individual (thermal, ohmic, ultrasounds and high hydrostatic pressure) treatments and combined (ohmic-thermal, high pressure–thermal and ultrasounds-thermal) treatments, on a kinetic basis, as prerequisites to produce paraprobiotics. The data were fitted to a probabilistic model, meaning that the death of viable cells is more probabilistic rather than deterministic events (Zhu et al., 2023). Therefore, the Weibull model developed by Peleg was used to estimate the shoulder and tailing in the inactivation rates. Given that the studies sustaining the attainable health benefits of paraprobiotics are limited, in our study the selected non-viable cell suspensions were tested for the cytocompatibility and antiproliferative effects, from the perspective of the safety use of the killed bacteria in different formulations.

2. Material and methods

2.1. Materials

The strain *Lactiplantibacillus plantarum* MIUG BL21 (*Lpb. plantarum* MIUG BL21) with probiotic potential (Cotârleț et al., 2023) part of the Microorganisms Collection of the *Dunărea de Jos* University of Galati, Romania (MIUG) was reactivated from the stock culture preserved by freezing, in 40 % solution of glycerol (*w/v*), at temperature of −80 °C was used in this study. Man Rogosa Sharpe (MRS) was purchased from Merck (Germany).

2.2. Preparation of bacterial cells

Lpb. plantarum MIUG BL21 cells were grown, by transferring 2 mL of stock culture into 9 mL MRS liquid for 48 h at 37 °C. Then, the culture (10 μL) was scarified on the MRS agar enriched with 30 g/L $CaCO_3$ (Yang et al., 2021), in order to obtain single colonies. Further, a single colony was transferred to liquid MRS (50 mL) and cultured in a stationary system for 48 h at 37 °C. The cells pellet was centrifuged at 7000 × *g* at 4 °C for 10 min, and the separated biomass was washed 3 times with phosphate buffer solution (PBS, pH 7.2), (Barros et al., 2021; Almada et al., 2021) and centrifuged under the same conditions as presented previously. The concentration of probiotic cells was measured by means of optical density at the wavelength $\lambda = 600$ nm, corresponding to a cell concentration of 1×10^8 colony forming units (CFU)/mL suspension.

2.3. Thermal treatment (HT)

Aliquots of 3 mL of cell suspension were transferred into sterile 15 mL Falcon tubes and heated from 60 °C to 100 °C, for 0–60 min, using a water bath (Julabo ED-5, Seelbach, Germany). At the end of heating, the suspensions were cooled quickly in an ice bath, to prevent further inactivation.

2.4. Ohmic treatment (OT)

A volume of 150 mL cell suspension was transferred to a sterile glass beaker and subjected to OT at different electric field magnitudes (4–55 V/cm) and holding time (2–25 min), in order to influence the electrical effects on the cells. An experimental discontinuous OT installation described by Nistor et al. (2015) was used. The voltage gradient variation was possible by modifying the distance between the electrodes. After OT, the suspensions were transferred in ice bath, to prevent further inactivation.

2.5. High hydrostatic pressure treatment (HPP)

The suspensions of probiotic cells (2 mL), distributed in sterile Eppendorf tubes, were subjected to HPP (300, 400, 500 MPa), for certain time (5–30 min at 300 MPa, 3–18 min at 400 MPa and 2–12 min at 500 MPa), at constant temperature of 8 °C. HPP experiments were conducted in four vessels (100 mL) in a laboratory-scale equipment (Resato, Roden, The Netherlands, 2011), using a mixture of water and propylene glycol (TR15, Resato) as pressurizing liquid. The compression rate was 200 MPa/min, and 1 min supplementary equilibration time was considered in all experiments. During the HPP treatment, the adiabatic heating increased temperature in the surrounding liquid to 13 ± 3.8 °C at 300 MPa 15 ± 4.6 °C, 17 ± 5.3 °C at 400 MPa and 25 ± 3.8 °C at 500 MPa. The decompression time of the vessels was approximately 0.5 s. After treatments, the suspensions were cooled quickly in an ice bath, to prevent further inactivation.

2.6. Ultrasound treatment (US)

A sonicator bath model (MRC Scientific Instruments) was selected as a low-intensity US process with a frequency of 30 kHz, pulse 1 and a power of 100 W/mL for this study. A volume of 15 mL of probiotic cell suspension was transferred into a sterile Falcon tube and subjected to US treatment for a holding time of 15 min at pulse amplitude of 20–100 %. Then, the suspensions were cooled quickly in an ice bath, to prevent further inactivation.

2.7. Combined ohmic and thermal treatment

The cells suspension (150 mL), distributed in sterile beakers, was initially subjected to OT, in a voltage gradient of 20 V/cm for 15 min, as described above. After the OT, 3 mL of the suspension were transferred

into sterile Falcon tubes and HT in a water bath (Digibath-2 BAD 4, Raypa Trade, Barcelona, Spain), at temperatures ranging from 50 to 70 °C, for different holding times (0–18 min). After the HT, the suspensions were rapidly cooled in an ice bath.

2.8. Combined high pressure and thermal treatment

The combined HPP with HT was carried out at 600 MPa, at a temperature of 50 °C equivalent to a temperature of 75 °C (after adiabatic heating) for 5 min.

2.9. Combined ultrasound and thermal treatment

The cells suspension (100 mL) was transferred in sterile beakers and subjected to US treatment for 30 min at power 100 W, frequency 30 kHz, and pulse amplitude 100 %. Further, volumes of 3 mL were transferred into sterile Falcon tubes, and subjected to HT in a water bath varying from 65 °C to 85 °C for 0–9 min. After the HT, the suspensions are cooled quickly by keeping them in an ice bath.

2.10. Viable cells count

Aliquots of 1 mL from the (un)treated cell suspensions were collected and homogenized with 9 mL of sterile physiological serum (0.9 % NaCl), in serial decimal dilutions. A volume of 1 mL from the appropriate dilutions were inoculated on a Petri dish containing MRS agar, supplemented with 30 g/L $CaCO_3$ (Yang et al., 2021). At least 2 plates for each dilution were prepared. Probiotic cultures were incubated at 37 °C for 72 h (Alamanda et al., 2021). The colonies were counted by the Koch method, as described by Michelutti et al. (2020).

2.11. Modelling of the Lpb. plantarum MIUG BL21 survival curves

To describe inactivation kinetics of *Lpb. plantarum* MIUG BL21, survival curves were fitted by the following decimal logarithm form of the Weibull model version (Eq. (1)) proposed by Peleg and Cole (1998).

$$\log_{10}\frac{N}{N_0} = -b \times t^n \quad (1)$$

Where: N is the number of viable cells after treatment (CFU/mL), N_0 is the initial number of viable cells (CFU/mL), b represents the rate coefficient, n characterizes the shape of the curves (dimensionless) ($n > 1$ produces convex inactivation curves, whereas $n < 1$ describes concave inactivation curves), and t is the holding time (minutes) (Buzrul, 2022). The mathematical expression of the Weibullian model proposed by Peleg is mathematically identical with the classical Weibull model, but it is statistically different. Both classical Weibull and the Peleg models are used for non-linear survival curves, whereas the Peleg version is more suitable to predict survival curves under dynamic conditions (Buzrul, 2022).

2.12. Cytotoxicity analysis of the probiotic inactivated cells suspensions

Prior to analysis, the following codification of the inactivated probiotic cells suspensions was used: $P1_{HT}$ – paraprobiotics obtained by HT at 90 °C for 20 min, $P2_{OH}$ – paraprobiotics obtained by combined OH – HT (20 V/cm followed by heating at 75 °C for 15 min), $P3_{HPP}$ – paraprobiotics obtained by HPP (600 MPa for 5 min) and $P4_{UH}$ – paraprobiotics obtained by combined US – HT (30 kHz and a power of 100 W/mL, amplitude pulse of 100 % for 30 min, followed by heating at 85 °C for 20 min).

The cell viability test was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), by evaluating the cellular metabolic activity through the ability to reduce a tetrazolic dye by mitochondrial dehydrogenases. For *in vitro* cytotoxicity analysis of the treated suspensions, the experiments were carried out on the stabilized murine fibroblast cell line NCTC, clone L929, from the European Collection of Cell Cultures (ECACC). The cells were maintained in Minimum Essential Medium (MEM) supplemented with 10 % fetal bovine serum and 1 % antibiotics (penicillin, streptomycin and neomycin) at 37 °C in a controlled atmosphere, containing 5 % CO_2. The samples were used after an initial filtration through filters with a porosity of 0.22 μm and sequential diluted in MEM supplemented with 10 % fetal bovine serum at different ratio (1:100, 1:50 and 1:10) (*v/v*). Further, the samples were incubated at 37 °C in a humid atmosphere with 5 % CO_2 for 72 h. NCTC cells were seeded in 96-well culture plates at a cell density of 5×10^3 cells/well for 24 h and allow them to adhere, then the culture medium was removed and replaced with the diluted inactivated cell suspensions. After 24 and 48 h of incubation, the culture medium was removed, the cells were washed with PBS (0.2 M, pH 7.4), followed by addition of 100 μL of 0.25 mg/mL MTT solution. After 3 h of incubation at 37 °C, the medium was removed and 100 μL isopropyl acid was added. The plates were kept at room temperature for 15 min with gentle shaking to ensure uniform color distribution, followed by absorbance reading at 570 nm (Tecan plate reader Sunrise, Tecan, Austria). The results were reported as percentage of viability, by comparing with the control sample (untreated cells), considered at a viability level of 100 %. All samples were tested in triplicate.

2.13. Antiproliferative activities

The *in vitro* antiproliferative experiments were performed on the stabilized cell line HT-29 derived from human colorectal adenocarcinoma (The European Collection of Authenticated Cell Cultures, ECACC). The cells were preserved in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum and 1 % antibiotics (penicillin, streptomycin and neomycin) at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. The samples were filtered and diluted as described above. The cell viability test was performed using the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The optical density of the formazan solution was measured spectrophotometrically, and the values obtained are directly proportional to the number of living cells at the end of the incubation. HT-29 cells were seeded in 96-well culture plates at a cell density of $5x10^3$ cells/well for 24 h to allow to adhere, followed by culture medium removing and replacing with the diluted probiotic inactivated cell suspensions. After 24 h and 48 h of incubation, the culture medium was removed, the cells were washed with PBS (0.2 M, pH 7.4), followed by addition of 100 μL MTT (0.25 mg/ml) solution. After 3 h of incubation at 37 °C, the medium was removed and 100 μL isopropyl acid was added. The plates were kept at room temperature for 15 min with gentle shaking for color uniformization, after which the absorbance was measured at 570 nm (Tecan Sunrise plate reader, Tecan, Austria). The results were reported as percentages of cells viability reported against control sample (untreated cells), considered 100 % viable. All samples were tested in triplicate.

2.14. Cells morphology

For both cellular lines, the cell morphology was highlighted using Giemsa staining. The cells were seeded in 24-well culture plates at a cell density of 5×10^4 cells/mL for 24 h, followed by addition of the diluted probiotic inactivated cell suspensions and subsequently incubated under standard conditions for 48 h. After removing the culture medium, the cells were washed with PBS (0.2 M, pH 7.4) and fixed with cold methanol (−20 °C) for 5 min. After removing the methanol, the cells were washed with distilled water and stained with Giemsa solution for 20 min. After washing with distilled water, the cells were examined with a Zeiss Axio Observer optical microscope (20× objective).

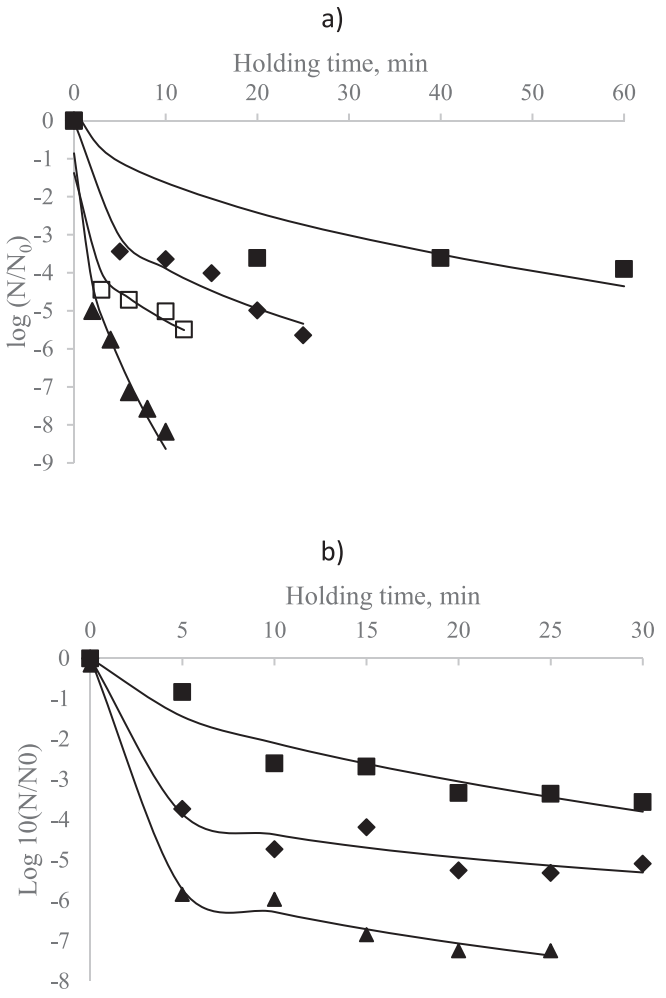

Fig. 1. Survival curves of *Lactobacillus plantarum* MIUG 21 during thermal treatment (a) (■60 °C, ◆70 °C, □80 °C, and ▲90 °C) and high pressure (b) (■300 MPa, ◆400 MPa and ▲500 MPa). Data were fitted with Weibull and are the average of three counts. The standard errors for the survival data were less than 0.50 $\log_{10}$.

2.15. Cells membrane integrity

The cells membrane integrity was evaluated based on the amount of the lactate dehydrogenase (LDH) released in the medium after treatment with probiotic inactivated cells, due to the cell damage. The plasma membrane of the cells is impermeable to the LDH enzyme, and the release of the enzyme from cytosol into the medium, indicates damages in the cell membrane and, thus a decrease in cell viability. For the LDH dosage, the *in vitro* Toxicology Assay Kit, Lactic Dehydrogenase based – TOX7 (Sigma-Aldrich) was used. After 24 and 48 h of incubation of the cells in the presence of the probiotic cell's suspensions, 50 μL of medium were transferred to a plate with 96 wells. Later, 100 μL of reaction mix (consisting of equal parts of substrate, cofactor and dye) and probiotic cells were added and incubated at room temperature, in the dark, for 30 min. Further, 15 μL of 1 N HCl solution was added, and the absorbance (OD_{sample}) was measured at wavelength of 490 nm (Tecan Sunrise plate reader, Tecan, Austria), using a reference wavelength of 690 nm. The results were reported in arbitrary units (a.u.), according to Eq. (2):

$$LDH\ release = \frac{OD_{sample}}{OD_{control}} \quad (2)$$

The corresponding value for the control (untreated cells) is considered 1. The measured OD values are directly proportional to the number of cells that have lost their cell membrane integrity. All samples were worked in triplicate.

Table 1
The kinetic parameters using the Weibull version proposed by Peleg for *Lpb. plantarum* MIUG 21.

Temperature (°C)	*b*	*n*	R^2	RMSE
Thermal treatment				
60	2.86 ± 0.10	0.07 ± 0.01	0.99	0.227
70	1.75 ± 0.42	0.34 ± 0.06	0.96	
80	3.74 ± 0.29	0.14 ± 0.03	0.99	
90	3.86 ± 0.53	0.32 ± 0.09	0.99	
High pressure (MPa)				
300	0.60 ± 0.26*	0.54 ± 0.14	0.92	0.104
400	3.17 ± 0.17	0.17 ± 0.05	0.96	
500	4.16 ± 0.38	0.22 ± 0.04	0.99	
Ultrasounds combined with thermal treatment				
65	2.21 ± 0.30*	0.26 ± 0.07	0.99	0.334
75	3.57 ± 0.10	0.15 ± 0.01	0.93	
85	3.00 ± 0.11	0.47 ± 0.04	0.99	

The results are expressed as average values for two determinations ± SD.

2.16. Statistical analysis

The data were obtained from independent experiments with repetition, and the means were obtained from the triplicates. For modelling the kinetic inactivation of probiotic cells, a modified version of Weibull model version, proposed by Peleg, was applied using SAS Windows 9.0 program (Cary, NC, USA). The statistical significance was assessed by using analysis of variance (ANOVA) followed by posthoc analysis with Tuckey test, when appropriate ($p < 0.05$). The statistical analysis was carried out using Minitab 19 statistical software (Minitab LLC, State College, PA, USA). Stochastic simulations of the "5D" decimal reduction time were performed for the estimated parameters variations using Weibull model version proposed by Peleg (Eq. (1)), based on Monte Carlo simulation (1000 iterations) using Excel software (Microsoft® Excel® LTSC MSO).

3. Results and discussion

3.1. The kinetics of Lpb. plantarum MIUG BL21 inactivation

Many new genera, such as *Lactiplantibacillus* spp. such as *Lpb. plantarum* and *Lpb. paraplantarum* are accepted as probiotics, if their daily intake is at least 1×10^9 CFU per day (Djukić-Vuković et al., 2021). The probiotic biomass with lower viability or inactivated cells may bring beneficial effects on health, when administered as food or nutraceuticals (Sanders et al., 2019). In this study, the probiotic *Lpb. plantarum* MIUG BL21 strain was inactivated using different techniques in order to produce paraprobiotics. For each treatment used, the results were expressed as the logarithmic reduction of the number of culturable cells after selected treatments, in relation with the initial number of bacterial cells. The logarithmic decrease in cells number, as a function of each HT and HPP treatments is shown in Fig. 1. The goodness of the fit was evaluated in terms of R^2 and root mean-square deviation (RMSE) as showed in Table 1. It can be seen that Weibull model version proposed by Peleg provided a good fit of the microbial inactivation for all treatments applied in this study (Fig. 1 and Table 1).

Regarding the HT inactivation, as presented in Fig. 1(a), an increase of the inactivation rate can be noticed with the increase in temperature from 60 to 90 °C. As such, the decrease of the survival rate ranged from 3.90 log N/N_0 after 60 min of treatment at 60 °C to 8.18 log N/N_0 after 10 min at 90 °C. Barros et al. (2021) suggested that *Lactobacillus (L.)*

acidophilus was completely inactivated after a treatment at 95 °C/5 min, while *L. casei* was more heat-resistant, being completely inactivated at 95 °C/7 min. Therefore, the inactivation parameters are strictly dependent on the probiotic strain characteristics, and thus a different time and temperature for the complete inactivation of each probiotic may be required (Ou et al., 2011). The Weibull model version proposed by Peleg allowed to estimate the kinetic parameters, based on Eq. (1). Regardless of the treatment applied, the shape parameter *n* was lower than 1, indicating the resistance of the *Lpb. plantarum* MIUG BL21 to inactivation at higher exposure time, observed as a tailing of the inactivation curves (Fig. 1). Moreover, it can be seen that *n* was independent of temperature, results that is in agreement with previous reported studies (Buzrul, 2022; van Boekel, 2002). On the other hand, *b* coefficient showing the inactivation rate, was temperature dependent in the 70 to 90 °C temperature range. Initially, at temperature of 60 °C and 70 °C, the *b* coefficient decreased by 1.63-fold, while in the temperature range of 70–90 °C, *b* coefficient increased by 2.21-fold, reaching a maximum of 3.86 ± 0.53, thus indicating a faster inactivation at higher temperatures. Based on the results presented in Fig. 1 and Table 1, and considering the variation of surviving cell numbers, but also the inherent variation of the individual parameters resulted from Peleg model, a Monte-Carlo simulation was conducted with 1,000 iterations to obtain a "5D" inactivation of the probiotic. The results indicated the average time for 5 Logs inactivation at 90 °C of 10.05 min and a probability of 98.9 % to reach less than 25 min for "5D" inactivation in a total of 1,000 experiments. Other researchers have also considered the usefulness of establishing 5 Logs as a target for inactivation (Buzrul, 2022). Our results are in good agreement with those reported by Ding & Shah (2007), who suggested that exposure of probiotic cells at 65 °C for 30 min, allowed a decrease in the living cells ranging from 3.6 to 5 Logs of CFU/mL for six probiotic cultures. In these conditions, *D* values defined as the time required to generate a 1 log loss of viability were estimated at 6–10 min, which are in line with those of Mandal et al. (2006), who suggested a $D_{65°C}$ value of 3 min for *L. casei*.

In terms of OT, the analysis of survival data performed at different voltage gradients (varying from 4 to 55 V/cm), showed that, regardless voltage, no complete inactivation was achieved. A decrease in survival rate of 2.07 log N/N_0 was found for a voltage gradient of 20 V/cm, after 15 min of treatment. The incomplete inactivation of probiotic cells may be explained by the different extent of the membrane permeabilization and damages, due to different voltage. The effect was explained by Almeida et al. (2019) and Díaz et al. (2010), who suggested that the increase in damaged cells may not occur at the same extent, despite the strong contribution of the membrane potential to homeostasis, the isolated reduction in membrane potential does not necessarily reflect cell death. Further, in order to enhance the inactivation, the OT was combined with further HT at two selected temperatures (70 °C and 90 °C). The combined OT and HT allowed the complete inactivation of the probiotic cells, at a voltage gradient of 20 V/cm for 15 min, followed by a HT at 70 °C for 15 min or at 90 °C for 5 min. However, when estimating the kinetic parameters, the Weibull model version proposed by Peleg did not allow a reasonable fit of the experimental values to the predicted data, thus the estimation of the kinetic parameters was not possible. Barros et al. (2021) suggested an optimal ohmic treatment inactivation at 8 V/cm for paraprobiotics production, allowing to maintain the probiotic properties.

For HPP treatments, the results showed that by varying the pressure at a constant time (10 min), an increase in the inactivation degree from 1.18 log N/N_0 at 100 MPa to 6.62 log N/N_0 at 600 MPa was found (data not shown). Further, inactivation kinetics studies driven by pressure increase between 300 and 500 MPa for preset times (0–30 min), showed a significant decrease in log N/N_0 up to 6.5 after 30 min at 400 MPa and to 7.08 after 10 min at 500 MPa. Fig. 1(b) indicates a good accuracy of the Weibull model version proposed by Peleg to describe the experimental results, allowing to estimate the kinetic parameters (Table 1). Similar to the thermal treatment, the *n* coefficient was lower than 1, while the *b* coefficient increased with increasing pressure, with a highest value of 4.16 ± 0.38 obtained at 500 MPa. The experimental results indicated a complete inactivation of the probiotic cells for paraprobiotics production, at 600 MPa, temperature of 8 °C, for a holding time equal or higher than 10 min. A Monte Carlo simulation of 1000 iterations took into account the variation of surviving cell numbers, but also the inherent variation of the individual parameters resulted from Peleg model (Eq. (1)), to obtain a "5D" inactivation of the *Lpb. plantarum* MIUG BL21 at 500 MPa. The results indicated the average time for 5 Logs inactivation of 3.08 min at 500 MPa, and a probability of 99.7 % to reach less than 12 min for "5D" considering a total of 1,000 experiments. The same effect of total inactivation was ensured after combined mild thermal and high-pressure treatment at 600 MPa and 50 °C after 5 min of treatment. Tsevdou et al. (2020) studied the effect of HPP processing on the viability of *Bifidobacterium bifidum* and *L. casei* in different pH values (6.5 and 4.8) model systems, on a kinetic basis. The loss in cells viability as a function of pressurization time at different combinations of pressure (100–400 MPa) and temperature (20–40 °C) was described by first order kinetics, allowing to estimate the *D* values of 44.5 ± 6.39 min at pH 4.8 and a significant pressure resistance at pH 6.5, corresponding to a *D* values of 281 ± 19.7 min. Comparable results were obtained where more than 5 Log inactivation was registered after 30 min treatment at 400 MPa, however, a non-linear inactivation kinetics described the inactivation of *Lpb. plantarum* MIUG BL21, as opposed to linear inactivation of *L. casei*. Moreover, in mixed carrot and mango and carrot juice, *L. plantarum*, survived after treatment at 600 MPa for 5 min at 25 °C (Oliveira et al., 2020). In our case, a complete inactivation was possible after the treatment at 600 MPa for 10 min at 8 °C.

The US treatment was performed by varying the impulses amplitude values (from 20 % to 100 %), under constant conditions of power (100 W), frequency (30 kHz), and holding time (15 min). The inactivation mechanism of US treatment involves breaking of the cell wall, thus disintegrating the cell membrane and DNA (de Almada et al., 2016), with or without a lethal effect of cells, depending on the energy level, which is a function of both the net power and the exposure time (Jomdecha and Prateepasen, 2010). The obtained results showed a maximum reduction in viable cells of 3.6 log N/N_0 after US treatment for 10 min at 100 % amplitude. Costa et al. (2013) applied US treatment to pineapple juice containing *L. casei* NRRL B442 at higher intensity of 376 W/cm^2 for 10 min, at 500 W and frequency of 19 kHz, demonstrating that the survived cells have maintained their viability after 48 days of storage under refrigerated conditions. Given the fact that US treatment is not able to inactivate completely the probiotic cells, further experiments were carried by combining US treatment (at amplitude of 100 % for maximum 30 min) with HT at different temperature–time combinations (Table 1). A 5.0 Log decrease was observed after combining US treatment for 18 min with thermal treatment at 85 °C for 20 min.

Overall, analyzing the kinetic parameters showed in Table 1, it can be hypothesized that in all the tested treatments, the inactivation curves were concave, suggesting that with increasing holding time, the surviving cells are more resistant and require longer time exposure for complete inactivation (Peleg, 2021). The Monte Carlo simulation based on 1000 iterations was performed using Peleg model (Eq. (1)) for the US treatment (power 100 W, frequency 30 kHz, pulses 1 s, duration 15 min) followed by a HT at 85 °C for 20 min. The results indicated the average time for *Lpb. plantarum* MIUG BL21, 5 Logs inactivation of 3.02 min, for the HT subsequent to the US treatment and a probability of 99.9 % to reach less than 5 min for "5D" considering a total of 1,000 experiments.

3.2. Cytocompatibility assay for the inactivated probiotic suspension and LDH release

Cytocompatibility of paraprobiotics are related with the ability of cells components placed in direct contact with the cellular component of vital tissues, to express physiological levels of proliferation, migration, and survival. In this context, in order to evaluate the cytocompatibility

Table 2
Cells viability in the presence of different concentrations of paraprobiotics by MTT assays after 24 h and 48 h of incubation.

Coded samples (concentration)	Cells viability (%), Murine fibroblast cell line NCTC, clone L929					
	24 h			48 h		
	1 mg/mL	2.5 mg/mL	5 mg/mL	1 mg/mL	2.5 mg/mL	5 mg/mL
$P1_{HT}$	103.42 ± 4.40	103.56 ± 3.98	103.46 ± 3.94	99.68 ± 2.48	104.68 ± 2.82	98.60 ± 3.69
$P2_{OH}$	110.00 ± 3.92*	106.78 ± 7.69	100.90 ± 3.67	111.76 ± 1.88*	116.09 ± 1.16*	97.78 ± 3.88
$P3_{HPP}$	109.40 ± 6.73	90.11 ± 4.03	66.09 ± 1.70*	89.93 ± 9.93	74.38 ± 5.23*	39.08 ± 2.85*
$P4_{UH}$	112.00 ± 4.55*	94.94 ± 6.35	88.07 ± 2.71*	101.34 ± 7.36	94.10 ± 7.51	83.35 ± 9.30
Coded samples (concentration)	**Cells viability (%), HT-29 derived from human colorectal adenocarcinoma**					
	24 h			**48 h**		
	1 mg/mL	2.5 mg/mL	5 mg/mL	1 mg/mL	2.5 mg/mL	5 mg/mL
$P1_{HT}$	114.87 ± 3.73*	112.33 ± 4.03*	102.31 ± 1.82	119.06 ± 0.52*	106.87 ± 1.85*	105.09 ± 2.19*
$P2_{OH}$	116.96 ± 8.29*	109.34 ± 7.19	64.41 ± 4.22*	119.72 ± 4.37*	107.19 ± 3.04*	41.69 ± 3.48*
$P3_{HPP}$	76.65 ± 0.79*	73.51 ± 3.96*	53.16 ± 3.45*	74.06 ± 3.20*	67.11 ± 2.77*	28.12 ± 0.35*
$P4_{UH}$	84.91 ± 6.30*	84.54 ± 3.88*	75.44 ± 1.24*	82.12 ± 0.19*	78.14 ± 2.75*	70.83 ± 2.33*

* Significant differences between control and analyzed samples using Dunnett Multiple Comparison Test ($p < 0.05$). The control (untreated cells) to which all samples were referred is considered to have 100 % viability.

Table 3
Cells viability in the presence of different concentrations of paraprobiotics by LDH assay after 24 h and 48 h of incubation.

Coded samples (concentration)	Cells viability (%), Murine fibroblast cell line NCTC, clone L929					
	24 h			48 h		
	1 mg/mL	2.5 mg/mL	5 mg/mL	1 mg/mL	2.5 mg/mL	5 mg/mL
$P1_{HT}$	87.66 ± 0.59*	89.39 ± 1.96*	96.65 ± 3.44	91.76 ± 6.06	96.45 ± 1375	92.39 ± 10.94
$P2_{OH}$	86.70 ± 4.48*	90.69 ± 5.24	100.90 ± 4.11	86.93 ± 9.03	87.74 ± 14.26	107.76 ± 13.43
$P3_{HPP}$	107.12 ± 3.31	100.07 ± 5.65	134.19 ± 2.67*	109.10 ± 5.45	125.30 ± 15.21*	155.63 ± 6.82*
$P4_{UH}$	100.58 ± 7.79	105.97 ± 2.45	114.25 ± 4.98	99.85 ± 7.73	107.69 ± 9.42	117.62 ± 12.07
Coded samples (concentration)	**Cells viability (%), HT-29 derived from human colorectal adenocarcinoma**					
	24 h			**48 h**		
	1 mg/mL	2.5 mg/mL	5 mg/mL	1 mg/mL	2.5 mg/mL	5 mg/mL
$P1_{HT}$	93.16 ± 12.25	98.13 ± 15.13	105.50 ± 9.78	91.76 ± 6.06	96.45 ± 13.75	92.39 ± 10.94
$P2_{OH}$	104.77 ± 7.67	84.86 ± 1.19	158.50 ± 9.51*	105.59 ± 6.94	122.38 ± 12.18	206.87 ± 18.02*
$P3_{HPP}$	122.58 ± 6.19*	130.81 ± 7.48*	143.57 ± 10.82*	124.64 ± 9.82*	132.72 ± 6.21*	172.50 ± 6.18*
$P4_{UH}$	115.93 ± 5.33*	117.34 ± 2.44*	123.65 ± 5.80*	116.25 ± 4.28*	122.20 ± 2.75*	132.04 ± 6.39*

* Significant differences between control and analyzed samples using Dunnett Multiple Comparison Test ($p < 0.05$). The control (untreated cells) to which all samples were referred is considered to have 100 % viability.

of the probiotic-inactivated cells, the cytotoxicity induced by paraprobiotics was assessed by the MTT assay, after the murine NCTC fibroblast cell line, clone L929 cell cultures were incubated for 24 h and 48 h. The results showed a high level of biocompatibility for all the samples tested, up to dilution 1:10 (Table 2). Therefore, no distinctive reduction in the cell viability is observed even in high concentrations of paraprobiotics, which is as a result of its cytocompatibility, except for the $P3_{HPP}$ which presented a cell viability of 66.09 ± 1.70 % at dilution 1:10, after 24 h of incubation. However, the cell viability values varied between 90.11 ± 4.03 % and 112.00 ± 4.55 % after 24 h, and between 39.08 ± 2.85 % and 116.09 ± 1.16 % after 48 h of incubation (Table 2). Therefore, a stimulative effect on cells proliferation can be observed, even after 48 h of incubation, in a concentration dependent manner. However, after 48 h of incubation, cell proliferation was observed up to dilution of 1:50, for all the samples, except $P3_{HPP}$.

At low concentration, all the samples showed $LDH_{release}$ values close to 1, suggesting a low release of the enzyme in the medium, comparable with the control (Table 3). However, for the variants $P3_{HPP}$ and $P4_{UH}$, the LDH values were higher when compared to the control sample, suggesting a higher cytotoxicity, in a concentration dependent manner.

The optical microscopy images of NCTC murine fibroblasts cultured in the presence of *Lpb. plantarum* MIUG BL21 inactivated cells for 48 h, revealed a normal cellular morphology, similar to that of the control sample (Fig. 2). The cells showed a normal, slightly polygonal appearance, with 2–3 cytoplasmic extensions, fine cytoplasm and euchromatic nuclei with multiple nucleoli. The cell density was similar with the control sample. As expected, when diluted at 1:10, the paraprobiotics induced significant changes in cell morphology, decreasing the cell density, suggesting a toxic effect (Fig. 2).

The obtained results highlight that the cytocompatibility of paraprobiotics largely depends on the inactivation method. However, it has been suggested that the health-related benefits of paraprobiotics, such immunomodulatory properties and to modulate the intestinal microbiota, and bacterial translocation highly depends on the ability of inactivation treatment to preserve the cell components of interest, such as teichoic acid, lipoteichoic acid, peptidoglycan release, cell surface morphology and, and not ultimately, the cell as a whole unit (Mehta, Ayakar & Singhal, 2023).

3.3. Antiproliferative effect of the inactivated probiotic suspension and LDH release

It is well known that antiproliferative potential is relating to a substance used to prevent or retard the spread of cells, especially malignant cells, into surrounding tissues. Therefore, the antiproliferative effect of the inactivated samples was tested by MTT assay on the stabilized cell

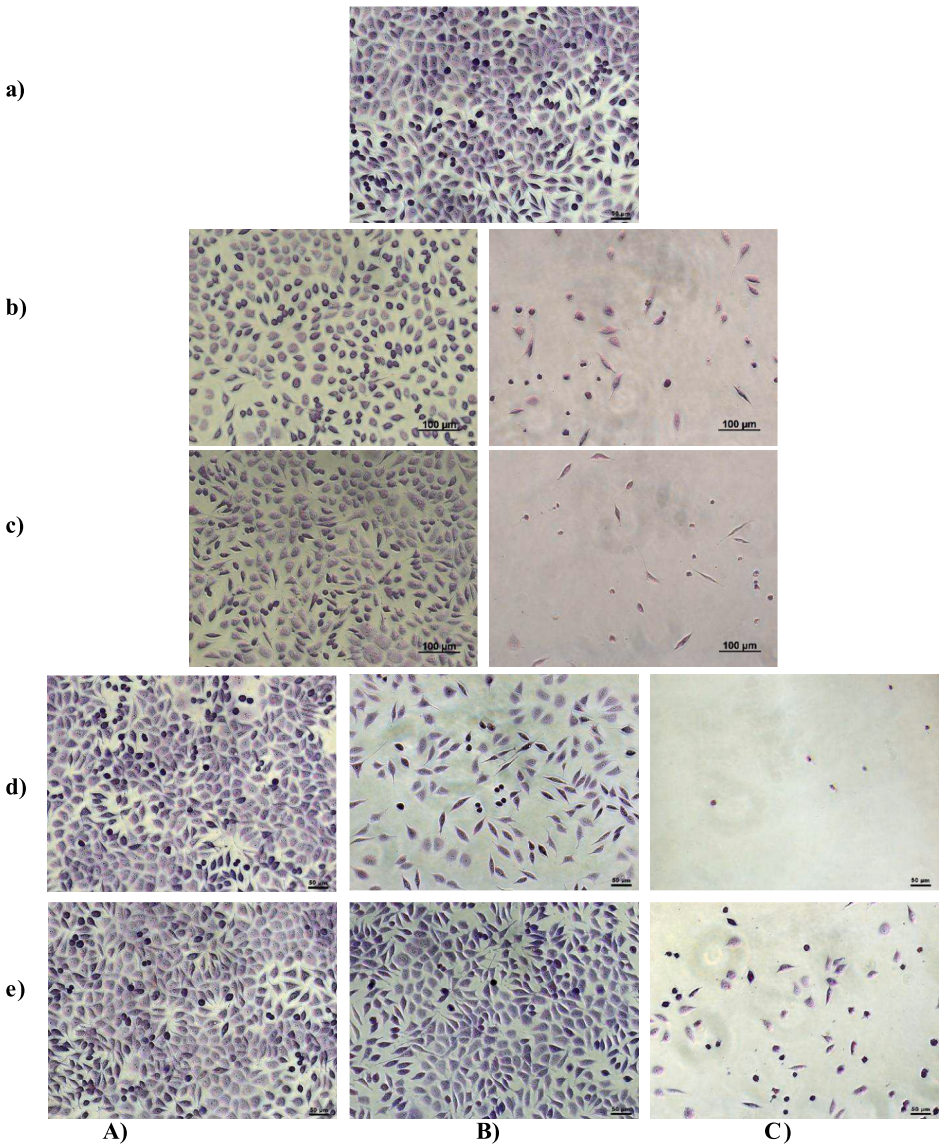

Fig. 2. Cytotoxicity of different concentrations of probiotic inactivated cells (a – control, b – paraprobiotics obtained by thermal treatment (temperature 90 °C, 20 min), c – paraprobiotics obtained by combined ohmic heating – heat treatment (20 V/cm for 15 min, followed by heating at 75 °C for 15 min), d – paraprobiotics obtained by high pressure treatment (600 MPa for 5 min) and e – paraprobiotics obtained by combined ultrasound treatment – heat treatment (30 min, followed by heating at 85 °C for 20 min, A – dilution 1:100, B – dilution 1:50, C – dilution 1:10), using the NCTC fibroblasts, after 24 h of incubation.

line HT-29, derived from human colorectal adenocarcinoma. The results (Table 2) showed antiproliferative activities for samples $P2_{OH}$, $P3_{HPP}$ and $P4_{UH}$, with cytotoxic effects at the lower dilution used (1:10). Therefore, the sample $P3_{HPP}$ showed a significant decrease up to 53.16 ± 3.45 % cells viability after 24 h of incubation, while increasing the incubation to 48 h led to maximum viability of 28.12 ± 0.35 %. The samples $P1_{HT}$ and $P2_{OH}$ showed significant higher cells viability, when diluted at a ratio of 1:50, of more than 95 %, both after 24 h and 48 h of incubation (Table 2).

LDH release test highlighted the MTT assay results, with the samples $P2_{OH}$, $P3_{HPP}$ and $P4_{UH}$ expressing tumoral effects at the highest concentrations. For the samples $P1_{HT}$, no damages on membrane integrity were observed (Table 3). The optical microscopy images of intestinal tumor cells HT-29 cultured in the presence of probiotic inactivated cells, for 48 h, revealed a normal cell morphology, similar with control, at higher dilution used. Samples $P3_{HPP}$ and $P4_{UH}$ induced changes in the typical cuboidal epithelial morphology at the higher concentrations, with concomitant changes in cell shape by rounding and substrate detaching, and the appearance of vacuoles were observed (Fig. 3).

İncili et al. (2023) suggested that the freeze-dried paraprobiotic of *Pediococcus acidilactici* contained a wide variety of bioactive, including different concentration of organic acids, free amino acids, free fatty acids (short-, medium-, and long-chain fatty acids), polyphenols and some volatile compounds such as pyrazines, pyranone and pyrrole derivatives. The obtained results suggest that after inactivation, dead cells released bacterial components, such as cell-free supernatants (De Marco et al., 2018), exopolysaccharides (Liu et al., 2017), teichoic and lipoteichoic acids (Kim et al., 2017), peptidoglycans, LPS (Piqué et al., 2013), and different metabolites (De Marco et al., 2018), with cytotoxicity and antiproliferative effect in a dose-dependent manner.

However, the effects of inactivated cells suspension seem to be highly dependent on the type and intensity of the inactivation treatments. The present study supports the *in vitro* efficacy of non-viable probiotic cells. However, additional information is needed to phytochemical profiling of paraprobiotic suspensions, in order to establish the compound-function relationships.

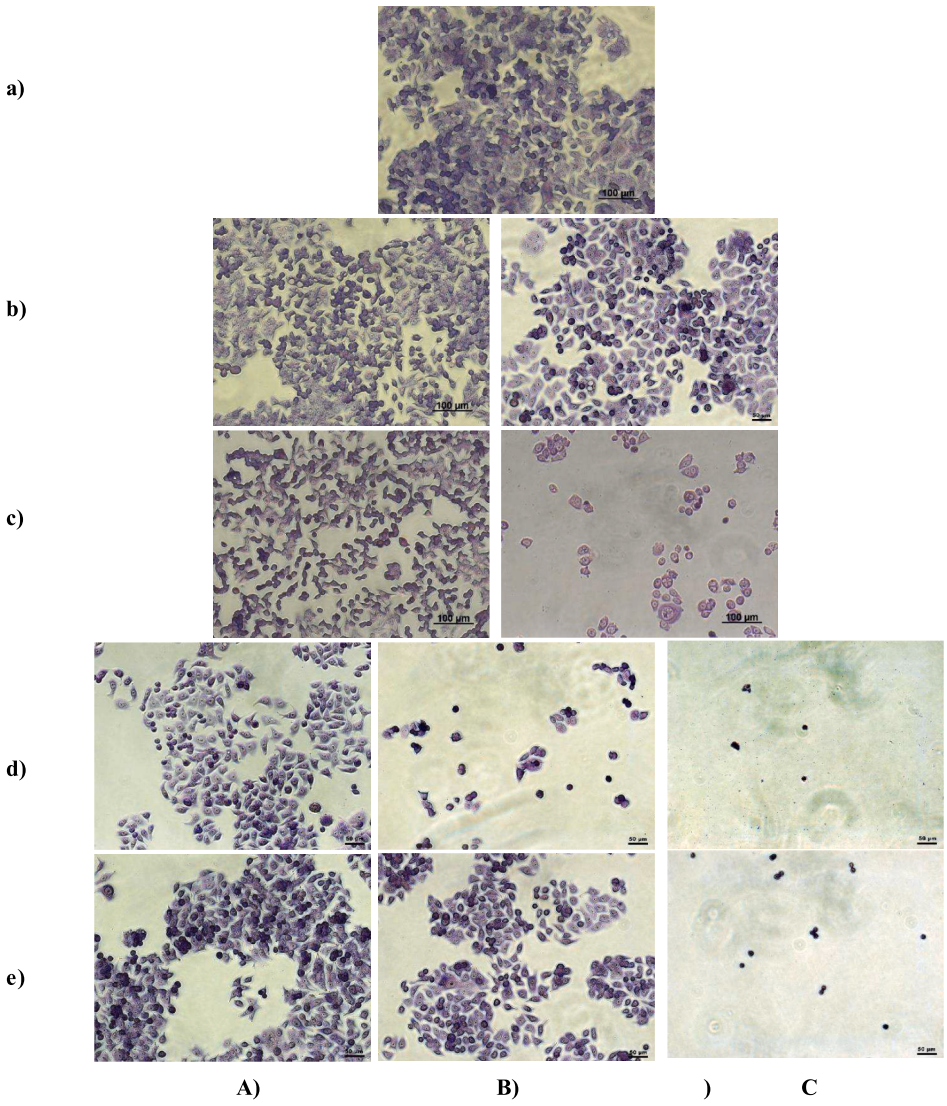

Fig. 3. Cytotoxicity of different concentrations of probiotic inactivated cells (a – control, b – paraprobiotics obtained by thermal treatment (temperature 90 °C, 20 min), c – paraprobiotics obtained by combined ohmic heating – heat treatment (20 V/cm for 15 min, followed by heating at 75 °C for 15 min), d – paraprobiotics obtained by high pressure treatment (600 MPa for 5 min) and e – paraprobiotics obtained by combined ultrasound treatment – heat treatment (30 min, followed by heating at 85 °C for 20 min, A – dilution 1:100, B – dilution 1:50, C – dilution 1:10), using the cell line HT-29 derived from human colorectal adenocarcinoma, after 24 h of incubation.

4. Conclusions

The results obtained in this study revealed a different behavior for inactivation patterns of *Lpb. plantarum* MIUG BL21 strain, previously selected as a lactic acid bacteria strain with probiotic potential, with thermal treatment allowing the highest decrease in survival rate, whereas complete inactivation was achieved by combining ohmic, high pressure and ultrasounds treatments with heating. The inactivation data were fitted to Weibullian model, allowing estimating the inactivation parameters. The complete inactivated cell suspensions were tested for cytotoxicity and antiproliferative activities. The non-viable cells suspensions showed cytocompatibility and cell proliferation properties at a higher diluted ratio, whereas the lactate dehydrogenase released test highlighted the tumoral effects for the samples inactivated by high pressure and ultrasound combined and thermal treatment at the highest concentrations tested. Further studies are needed in order to identify and quantify the paraprobiotics released as a function of inactivation treatments. These studies are currently developed in our laboratories.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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