DR. LILIA CAVAGLIERI (Orcid ID : 0000-0002-2380-906X)

Article type : Original Article

Cytotoxicity in Vero cells and cytokines analyses in Balb/c mice as safety assessments of the probiotic mixture *Saccharomyces cerevisiae* RC016 and *Lactobacillus rhamnosus* RC007 for use as a feed additive

Running headline: Safety of a probiotic mixture

A.S. Fochesato [a,b], M.P Martínez [a,b], F.S. Escobar [a,c], G.García [a,b], C.A. Dogi [a,c], L.R. Cavaglieri [a,c] *

[a] Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto. Ruta 36, km 601, (5800) Río Cuarto, Córdoba, Argentina.

[b] Fellow of Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina,

[c] Member of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina.

Authorship

A.S. Fochesato: substantial contribution to conception and design or the acquisition and analysis of data.

M.P Martínez: substantial contribution and acquisition of data.

G.García: substantial contribution and acquisition of data.

F.S. Escobar: acquisition and analysis of data.

C.A. Dogi: critically revising the manuscript.

L.R. Cavaglieri: conception and design; critically revising the manuscript and approval of the final submitted version.

***Corresponding author**

Dr. Lilia Cavaglieri, Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta 36 km 601, 5800 Río Cuarto, Córdoba, Argentina.
Tel-Fax. 54-358-4676231
E-mail:lcavaglieri@exa.unrc.edu.ar

Significance and impact of the study: The administration of a mixed formulation with *Saccharomyces cerevisiae* RC016 and *Lactobacillus rhamnosus* RC007 in healthy mice expands the knowledge on the beneficial effects of the use of these probiotic microorganisms in mixture for the development of feed additives.

Abstract

The objective was to carry out cytotoxicity assays in Vero cells and cytokines analyses in Balb/c mice as safety assessments to evaluate the probiotic mixture (M) *S. cerevisiae* RC016 (Sc) and *L. rhamnosus* RC007 (Lr) for use as feed additive. Vero cells (10^4 cells $well^{-1}$) were exposed to *Sc* (2.08×10^7, 2.08×10^6; 2.08×10^5 cells mL^{-1}), *Lr* (8.33×10^7; 8.33×10^6; 8.33×10^5 cells mL^{-1}) and their M (1:1). *Sc* concentrations did not affect the Vero cells viability; in contrast, they were lower when exposed to *Lr* (*P* <0.0001). Vero cells showed increasing viability with M decreasing concentrations (91% viability with M2). Control BALB/c mice received only PBS and the others received the M. The IL-10, IL-6 and TNFα concentrations from intestinal fluid were analyzed and no significant differences were observed among treatments. The same occurred with the ratio between IL-10/TNF-α. Beneficial effects of probiotics are associated with the regulation of the excessive inflammatory response; it is desirable they can modulate the cytokines production only under pathological conditions. Here, M administration to healthy mice did not induce negative side effects and expands the knowledge about beneficial effects of using probiotic microorganisms in mixture for feed additives development.

Key words: safety assessments, cytotoxicity, Vero cells, cytokines, BALB/c mice, probiotic, *S. cerevisiae, L. rhamnosus*, feed additive.

Introduction

The use of probiotics in animal nutrition is intended to improve feed conversion, promote growth, and inhibit the growth of pathogenic bacteria (Markowiak and Śliżewska, 2018). Modulation of the immune system by probiotics is an important potential benefit in animal production systems. Epithelial cells in the gastrointestinal tract (GIT) mucosa are the first line of defense against pathogenic microorganisms (Martens *et al.* 2018). Anatomical structures, immunological secretions such as mucus, immunoglobulins, antimicrobial peptides, and the epithelial junction adhesion complex are combined to exert a defense function (Wang *et al.* 2018). Probiotics modulate the components of the immune system at the GIT level, protecting the host from different types of pathogenic antigens. Also, they affect both, innate and adaptive immunity. Incorporation of probiotics in animal feeding requires a careful evaluation of their efficacy and safety (Kupryś-Caruk *et al.* 2018). The growing popularity of probiotics in foods, and the lack of international consensus on the methodology to assess their efficacy and safety, have led the Food and Drug Administration (FAO) to promote the development of guidelines for the evaluation of probiotics in food (FAO, 2016).

It was recognized that the evaluation of the possible risks associated with the incorporation of microorganisms in animal feed should include the identification at the strain level. The current state of evidence suggests that probiotic effects are strain-specific. This particular strain should not be associated with infections in humans or animals or should not contain antibiotic resistance genes. It is recommended to evaluate the capacity to produce toxins or to cause hyperstimulation of the immune system in the host. According to Shanahan (2012), the safety assessment and information on a particular probiotic strain cannot be generalized to similar probiotics (even with a different strain of the same species), as each probiotic requires a safety assessment and the particular risk. The adverse effects and the severity of the effects of a probiotic could be context-specific and depend on the susceptibility (immunity) and the physiological state of the host. Therefore, probiotic strains considered safe under certain conditions may not be safe under other conditions. Other recommended steps include in vitro and in vivo assessments to demonstrate safety and the final functional characterization (FAO 2016).

The objective of the present work was to carry out cytotoxicity assays in Vero cells and cytokines analyses in BALB/c mice as safety assessments to evaluate the probiotic mixture *S. cerevisiae* RC016 and *L. rhamnosus* RC007 for its use as a feed additive.

Materials and methods

Saccharomyces cerevisiae RC016 was isolated from pig intestine. Morphological, biochemical and molecular characterization were conducted. The sequence comparisons were performed

using the basic local alignment search tool (BLAST) program within the NCBI database and submitted to GenBank (ID KF447149.1). Stock cultures were maintained at 80°C in 30% (v/v) glycerol. *Lactobacillus rhamnosus* RC007 was isolated from maize silage. Fermentation pattern (API 50 CHL test) and the 16S rRNA gene sequence were conducted for biochemical and molecular identification (Dogi *et al.* 2013). Stock cultures were maintained at 80 °C in 15% (v/v) glycerol. These strains are currently deposited in the culture collection of the National University of Rio Cuarto collection centre, located in Río Cuarto, Córdoba, Argentina.

Vero cells (ATCC CCL-76) were obtained from the Asociación Banco Argentino de Células (ABAC). Cells were propagated in minimal essential medium (MEM; Gibco, USA) supplemented with 8% fetal calf serum (FCS; Natocor, Argentina), 50 μg mL^{-1} gentamicin and 2 mmol glutamine (Sigma-Aldrich, Italy). Vero cell viability was measured by a neutral red absorption assay (NRU). Cells were plated in 96-well culture plates at 10^4 cell $well^{-1}$ and, after monolayer formation, exposed to three concentrations of 24 h culture *S. cerevisiae* RC016 (Sc1, 2.08 x 10^7; Sc2, 2.08 x 10^6; Sc3, 2.08 x 10^5 cell mL^{-1}), three concentrations of 24 h culture *L. rhamnosus* RC007 (Lr1, 8.33 x 10^7; Lr2, 8.33 x 10^6; Lr3, 8.33 x 10^5 cell mL^{-1}) and the mixture (M) of both cultures (1:1) (M1: Sc1 + Lr1; M2: Sc2 + Lr2; M3: Sc3 + Lr3).

All treatments were dissolved in maintenance medium (MEM supplemented with 2% FCS, 50 μg/mL gentamicin, and 2 mM glutamine). Cells treated with MM alone were used as controls. After incubation at 37°C for 3 h, the medium containing the dye was removed and the wells were washed twice with heated Phosphate Buffer Saline (PBS) (150 μL $well^{-1}$). The dye within the viable cells was released by extraction with a mixture of acetic acid, ethanol, and water (1:50:49). After the cultures were shaken for 10 min, the absorbance values were read at 540 nm in a microplate reader (Thermo Labsystems Multiskan MS). For comparisons, relative cell viability was expressed as the percentage of the NRU control group (%). Untreated cells were used as controls.

The working protocol and the used techniques comply with the regulations of the Subcommittee on Animal Bioethics under the Ethics Committee of Scientific Research, as established in Resolution 253/10 of the Superior Council of the National University of Rio Cuarto.

BALB/c mice (female, 5 weeks old, weighing 9.3 ± 0.9 g) were divided into two groups (n=6): control group (C) and mixture group (M). In C group, the animals received 0.1 mL PBS orally daily whereas in M group, according to the results obtained in the cytotoxicity assay, the animals received 0.1 mL M2 resuspended in PBS orally daily. The animals were housed in the Animal Facilities Center of the National University of Río Cuarto and were kept in an environmentally controlled room with 12 h light/dark cycles. After 10 d, the mice were sacrificed by cervical dislocation and samples of intestinal content were obtained.

The intestine contents were collected from small intestines with 1 mL PBS and immediately centrifuged at 5000 g for 15 min at 4°C. The supernatants were recovered and stored at −80°C until cytokine determination using Cytometric Bead Array (CBA) (BD Bioscience, San Diego, EE.UU.). The concentration of IL-10; IL-6 and TNFα from the intestinal fluid of each mouse was obtained and the results were expressed in relation to the protein concentration measured in the sample. The total protein content of the samples was determined using the Bio-Rad Protein Assay based (Bradford, 1976).

Values were expressed as the mean ± standard deviation (SD) of the data and were representative of two different experiments. The means obtained from the trials were analyzed by analysis of variance (ANOVA). Variables with significant differences were compared using the Fisher´s least significant difference test (LSD). The analysis was performed using Info Stat for Windows 2012 Version 2.03 (SPSS Inc).

Results and Discussion

The use of probiotics in animal feed continues to be a promising option, in the face of pressures to reduce the use of antibiotics as growth promoters, to profitably produce safe food, to reduce environmental contamination with zoonotic pathogens and to maintain health in animals. *Saccharomyces cerevisiae* RC016 and *L. rhamnosus* RC007 have separately shown promising candidates to include them in an animal feed additive formulation and thus improve the health and performance of animal production. Moreover, they have demonstrated to significantly reduce the mycotoxins on *in vitro* and *in vivo* studies (García *et al.* 2018, Poloni *et al.* 2020). To complement these results the cytotoxicity using Vero cells and their effect on the immune system of BALB/c mice were tested here.

Approval of probiotic strains for use in animal nutrition requires demonstration of the absence of cytotoxicity in cell models such as Vero cells (Singh *et al.* 2018). Although significantly different compared to the control treatment ($P < 0.0001$), exposure of Vero cell culture to the tested Sc concentrations maintained cell viability at values greater than 60% (Fig. 1). In contrast, the Vero cells viability percentages were significantly lower when exposed to *Lr* ($P < 0.0001$). Vero cells showed increasing percentages of viability with decreasing concentrations of the mixture reaching 91% viability with M2 mixture. Probably, the significant decrease that occurs in the presence of *Lr* is due to the production of lactic acid during the 24 h of incubation, since the morphology of the monolayer was not affected, detecting only a change in the pH of the culture (result not shown). The methodology to determine cytotoxicity is varied in literature but it is used as a method of selecting probiotic strains. In agreement with our results, Chimchang *et al.* (2016) selected lactic bacteria with probiotic and anticancer properties evaluating the cytotoxicity of $1x10^8$ cell mL^{-1}

against Vero cells and found some strains produced soluble factors with high cytotoxicity but not the bacterial sediment itself.

Products that claim probiotic effects must contain a sufficient number of viable cells, between 10^6 to 10^{10} CFU g^{-1} to confer efficacy (Champagne *et al.* 2011). In this study, the used probiotic cells concentrations were included within these values.

In vivo studies with experimental models demonstrate that modulation of the host immune response is one of the most important properties attributed to probiotics (Galdeano *et al.* 2007). Taking into account these considerations and the importance of maintaining animal health without the use of antibiotics, the effect of oral supplementation of the mixture by analyzing certain cytokines involved in maintaining gut homeostasis (IL-6, TNF-α, and IL-10) was analyzed. The results showed that no significant differences were observed among the different treatments studied (Fig. 2). The same occurred with the ratio between anti and pro-inflammatory cytokines in intestinal fluid (IL-10/TNF-α). In the present study, the animals were not exposed to harmful stimuli, such as pathogenic microorganisms or inflammation-causing agents (chemical or biological) that allowed evidence of modulation of the immune system; the daily administered dose of the mixed formulation alone did not produce immune stimulation. The beneficial effect of probiotics is also associated with the regulation of the excessive inflammatory response; therefore, it is important to note that the administration of the mixture *S. cerevisiae* RC016 and *L. rhamnosus* RC007 to healthy mice did not induce increases in proinflammatory cytokines. Overproduction of some cytokines can cause a pathological inflammatory response and it is also desirable that the administration of probiotics can modulate the production of these cytokines only under pathological conditions and not under normal conditions (FAO, 2016).

In the present work, it was shown that the administration of *S. cerevisiae* RC016 and *L. rhamnosus* RC007 mixture to healthy mice did not induce negative side health effects. The administration of a mixed formulation in healthy mice expands the knowledge about the beneficial effects of using these probiotic microorganisms in mixture for the development of feed additives.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgements

This study was supported by grants from SECyT-UNRC and PICT 3089/18.

References

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72(1-2), 248-254.

Champagne, C.P., Ross, R.P., Saarela, M., Hansen, K.F., Charalampopoulos, D. (2011) Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices. *Int. J. Food Microbiol*. 149, 185-193.

Chimchang, J., Theparee, T., Wongein, S., Trivirot, T., Tanasupawa, S., Taweechotipatr, M., Wongsatayanon B. (2016) Screening of potential probiotic lactic acid bacteria with anticancer properties. *J. Med. Assoc. Thailand Chotmaihet thangphaet* 99, S70-S78.

Dogi, C.A., Fochesato, A., Armando, R., Pribull, B., Souza, M.M.S., Coelho, I., Cavaglieri, L. (2013) Selection of lactic acid bacteria to promote an efficient silage fermentation capable of inhibiting the activity of *Aspergillus parasiticus* and *Fusarium gramineraum* and mycotoxin production. *J. Appl. Microbiol.* 114, 1650-1660.

FAO (2016) Probiotics in animal nutrition – Production, impact and regulation by Yadav S. Bajagai, Athol V. Klieve, Peter J. Dart and Wayne L. Bryden. Editor Harinder P.S. Makkar. FAO Animal Production and Health Paper N°179. Rome.

Galdeano, C.M., De Moreno de LeBlanc, A., Vinderola, G., Bonet, M.E., Perdigón, G. (2007) Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. *Clin. Vaccine Immunol.* 14, 485-492.

García, G.R., Payros, D., Pinton, P., Dogi, C. A., Laffitte, J., Neves, M., Oswald, I. P. (2018) Intestinal toxicity of deoxynivalenol is limited by *Lactobacillus rhamnosus* RC007 in pig jejunum explants. *Arch. Toxicol.* 92, 983-993.

Kupryś-Caruk, M., Michalczuk, M., Chabłowska, B., Stefańska, I., Kotyrba, D., Parzeniecka-Jaworska, M. (2018) Efficacy and safety assessment of microbiological feed additive for chicken broilers in tolerance studies. *J. Vet. Res.* 62, 57-64.

Markowiak, P., and Śliżewska, K. (2018) The role of probiotics, prebiotics and synbiotics in animal nutrition. *Gut Pathogens* 10, 21.

Martens, E.C., Neumann, M. Desai, M.S. (2018) Interactions of commensal and pathogenic microorganisms with the intestinal mucosal barrier. *Nature Rev. Microbiol.* 16, 457-470.

Poloni, V., Magnoli, A., Fochesato, A., Cristofolini, A., Caverzan, M., Merkis, C., Montenegro, M., Cavaglieri, L. (2020) *Saccharomyces cerevisiae* RC016-based feed additive reduces liver toxicity, residual aflatoxin B_1 levels and positively influences intestinal morphology in broiler chickens fed chronic aflatoxin B_1 contaminated diets. *Animal Nut.* S2405-6545(19)30190-8.

Shanahan, F. (2012) A commentary on the safety of probiotics. *Gastr. Clin. North America* 41, 869.

Singh, P., Medronho, B., dos Santos, T., Nunes-Correia, I., Granja, P., Miguel, M.G., Lindman B. (2018) On the viability, cytotoxicity and stability of probiotic bacteria entrapped in cellulose-based particles. *Food Hydroc.* 82, 457-465.

Wang J, Ji H, Wang S, Liu H, Zhang W, Zhang D and Wang Y. (2018) Probiotic *Lactobacillus plantarum* promotes intestinal barrier function by strengthening the epithelium and modulating gut microbiota. *Front. Microbiol.* 9:1953.

Figure captions

Figure 1. Viability of Vero cells exposed to *S. cerevisiae* RC016 cells (Sc1, 2.08 x 10^7; Sc2, 2.08 x 10^6; Sc3, 2.08 x 10^5 cell mL^{-1}), *L. rhamnosus* RC007 cells (Lr1, 8.33 x 10^7; Lr2, 8.33 x 10^6; Lr3, 8.33 x 10^5 cell mL^{-1}), and the mixture of both (1:1) (M1: Sc1 + Lr1; M2: Sc2 + Lr2; M3: Sc3 + Lr3) determined by neutral red absorption assay. Results are presented as percentage (mean ± SD, n = 4) ($P < 0.0001$).

Figure 2. Cytokine concentrations in small intestine contents. Cytokine concentrations in the small intestine content were determined by Cytometric Bead Array. The experimental group was the control group (C): animals received orally 0.1 mL of phosphate-buffered saline (PBS); mix group (M): animals received orally 0.1 mL of the mixture (1:1) of *L. rhamnosus* RC007 (1 x 10^6 cell mL^{-1}) and *S. cerevisiae* RC016 (1 x 10^7 cell mL^{-1}) resuspended in PBS. Results are expressed as the concentration of each cytokine in pg/mg of proteins: a) IL-6; b) TNF- α and c) IL-10. The ratio between IL-10 and the pro-inflammatory cytokine TNF-α was also analyzed (d). Each bar represents the mean ± SD (n = 6, from 2 independent experiments).

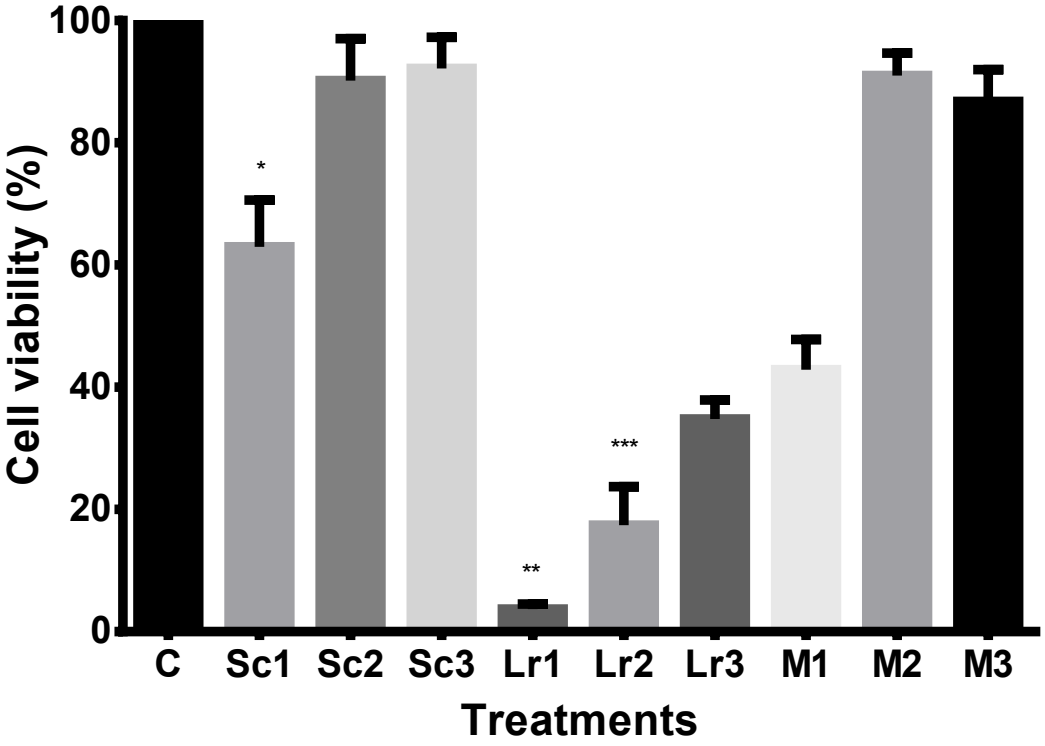

Figure 1.

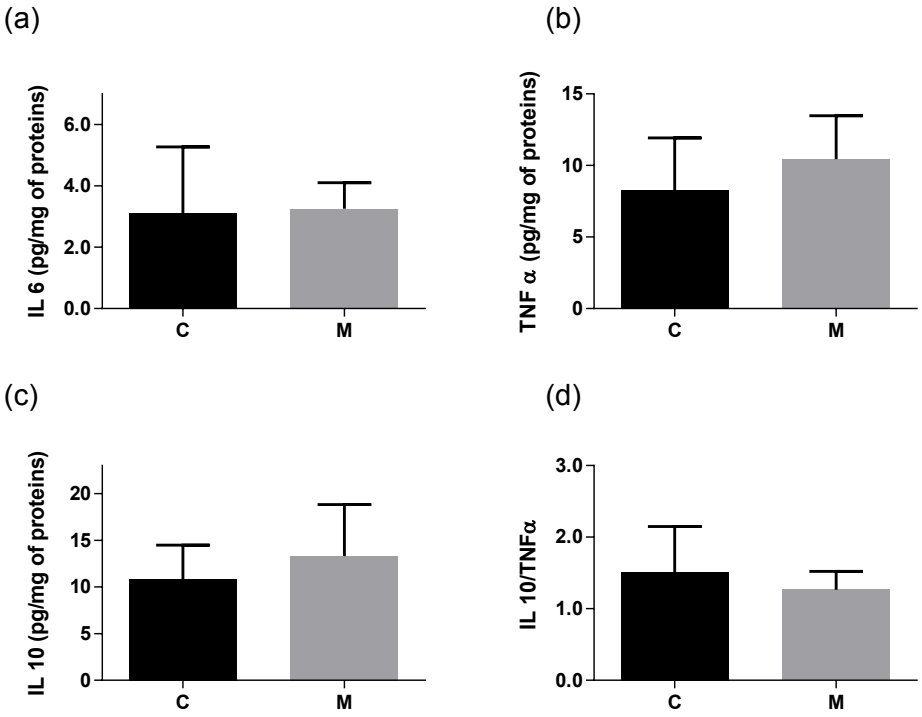

Figure 2.