

## Probiotic technological and functional characteristics of *Lactobacillus* strains isolated from chicken gut

[Características tecnológicas e funcionais de linhagens de *Lactobacillus* isoladas do trato gastrointestinal de frangos]

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### ABSTRACT

*Lactobacillus* spp. isolated from different portions of chickens' gastrointestinal tract were evaluated concerning their ability to survive in a water-in-oil (W/O) emulsion containing sesame and sunflower oil. After sixty days of emulsion storage under refrigeration, three of five strains tested survived in number equal to or higher than  $10^6$ cfu/g. *Lactobacillus reuteri* 2M14C, which presented the highest survival in W/O emulsion ( $10^7$ cfu/g), was tested for its capacity to resist throughout the passage through gnotobiotic mice gastrointestinal tract and for the ability to stimulate murine peritoneal macrophages phagocytosis. This strain remained at a number above  $10^9$ cfu/g feces during ten days of monoassociation, and monoassociated mice showed phagocytic activity significantly greater than the germ-free controls ( $P<0.05$ ). The results suggest that the formulation can be used to incorporate viable *Lactobacillus* spp. cells in animal feed. Moreover, the results suggest that *L. reuteri* 2M14C is a strong candidate to be incorporated in probiotic formulations for use in chicken.

Keywords: chicken, probiotic, *Lactobacillus*, emulsion, phagocytosis

### RESUMO

*Lactobacillus* spp. isolados de diferentes porções do trato gastrintestinal de frangos foram testados quanto à capacidade de se manterem viáveis em uma emulsão água/óleo (A/O) contendo óleos de gergelim e de girassol. Após sessenta dias de estocagem sob refrigeração, três de cinco linhagens testadas sobreviveram em concentração igual ou superior a  $10^6$ UFC/g. *Lactobacillus reuteri* 2M14C, que apresentou maior capacidade de sobrevivência na emulsão desenvolvida ( $10^7$ UFC/g), foi testado quanto à sua capacidade de sobreviver às condições do trato gastrintestinal in vivo em camundongos gnotobióticos. Após dez dias de monoassociação com *L. reuteri* 2M14C, foi testada também a capacidade de estimulação da atividade fagocítica de macrófagos peritoneais. A linhagem permaneceu em número superior a  $10^9$ UFC/g de conteúdo fecal durante os dez dias de monoassociação, e os camundongos monoassociados apresentaram atividade fagocítica maior ( $P<0,05$ ) que a do grupo controle isento de germe. Os resultados sugerem que a formulação proposta é capaz de manter a viabilidade de células de lactobacilos para adição em ração animal, necessitando, no entanto, de um acompanhamento dessa viabilidade por tempo maior de estocagem. Além disso, os resultados demonstram que *Lactobacillus reuteri* 2M14C é um forte candidato a ser adicionado em formulações probióticas para uso em frangos.

Palavras-chave: frango, probióticos, *Lactobacillus*, emulsão, fagocitose

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## INTRODUCTION

Probiotics are live microorganisms which when administered in adequate amount confer health benefits to the host (FAO/WHO, 2001). These beneficial effects include: immunomodulation, balance of colonic microbiota, anticarcinogenic effect, hypocholesterolemic effect, and antagonism against pathogens (Miyazaki *et al.*, 2010; Williams, 2010).

Scientific research supports the role of probiotics as effective alternatives to the use of antimicrobial growth promoters in animal nutrition (Mountzouris *et al.*, 2010), and the beneficial effects of probiotics on broilers have been reported (Higgins *et al.*, 2008; Eckert *et al.*, 2010). However, the screening of appropriate microorganisms must include technological and functional criteria in order to achieve probiotic efficacy. An essential feature for a probiotic is its ability to survive in sufficient number in the host gastrointestinal tract (Pitino *et al.*, 2010). Furthermore, this strain must be able to resist the stressing physicochemical conditions of the production process, to survive during the long-term storage period of the product that will carry it and to be produced in large quantities (Ross *et al.*, 2005). Also, a new candidate for use as a probiotic must not have any proven functional characteristic which cannot be modified by the production process.

Several attempts have been made to develop products containing probiotic microorganisms such as *Lactobacillus* and *Bifidobacterium* species for animal and human consumption (Williams, 2010). However, studies indicate that some probiotics may not survive and retain their activity in market preparations or in the host gastrointestinal tract, which limits their practical use in foods and pharmaceutical or food supplements (Stadler e Viernstein, 2003; Corcoran *et al.*, 2004). In this regard, the microencapsulation process of microbial cells with potential probiotic characteristics has been investigated in order to increase the viability of the microorganisms to ensure their passage through the host's gastrointestinal tract (Sultana *et al.*, 2000). Microencapsulation consists of bioactive materials and/or cells involved in polymeric ultrafine semi-permeable membranes to protect them from environmental damage without avoiding the passage of essential substances through the microcapsule (Orive *et al.*, 2004). Sesame oil (*Sesamum indicum* L.) has long been used for human consumption and it was demonstrated that encapsulation with artificial sesame oil emulsions

exert a significant protective effect on lactic acid bacteria in high acid gastric and bile salt simulated conditions (Hou *et al.*, 2003).

In our laboratory, *Lactobacillus* strains previously isolated from chicken gastrointestinal tracts have demonstrated probiotic characteristics (Souza *et al.*, 2007). Thus, one of the goals of this work was to evaluate if some of these *Lactobacillus* strains would be able to survive during production and storage in a water-in-oil (W/O) emulsion that has sesame oil as its encapsulating ingredient. In addition, it was evaluated if *L. reuteri* 2M14C, a strain that had the best survival capacity in the developed formulation, could survive at high population level in the gastrointestinal tract of monoassociated mice and stimulate their mononuclear phagocytic system. These characteristics could define this strain as a good probiotic candidate for chicken.

## MATERIAL AND METHODS

Germfree Swiss/NIH mice (Taconic, Germantown, NY, USA), 21-23 days old and of both genders were maintained at the Gnotobiology Laboratory of the Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil, according to the procedures described by Pleasants (1974). All experimental procedures were carried out according to the standards set forth in the "Colégio Brasileiro de Experimentação Animal" rules (COBEA, 2006). The study was approved by the Ethics Committee in Animal Experimentation of the Universidade Federal de Minas Gerais (CETEA/UFMG, protocol n°. 203/2009).

*Lactobacillus* strains used in this study were isolated from the gastrointestinal tract of free-range chickens and identified through the PCR-ARDRA methodology of intergenic spacers of 16S-23S rRNA (Mota *et al.*, 2006; Souza *et al.*, 2007). Five strains were used, *L. reuteri* 2M14C, *L. acidophilus* 4M14E, *L. vaginalis* 5M14E, *L. salivarius* 4C14C and *L. salivarius* 5C14C. They were activated twice in MRS broth (De Man, Rogosa and Sharpe, Difco, Sparks, MD, USA) at 37°C for 18h. Fifty milliliters of each active culture were centrifuged at 2000xg and washed with an equal volume of PBS (buffered saline solution, pH 7.3) two fold. After washing, each culture was resuspended in reconstituted nonfat dry milk 10% (NFDM 10%) in order to obtain a suspension with 10<sup>10</sup> colony forming units (cfu/ml) of lactobacilli that were incorporated into the emulsion.

From a previous standardization, a stable emulsion containing viable cells from each isolate was obtained with 1.06% of commercial sesame oil, 53.48% of commercial sunflower oil, 10.91% of glyceril monostearate, 6.36% cetostearic acid and 28.19mL of 10% NFDM. The total amount of sesame oil was heated at 70°C and after this time, sunflower oil, glyceril monostearate and cetostearic acid were added and heated at 70°C until the ingredients were completely mixed. This mixture was then cooled to 40°C. Adequate volume of 10% NFDM containing *Lactobacillus* spp. cells was heated at 40°C in a water bath and was slowly incorporated into the flask containing the other ingredients, followed by mechanical agitation (630rpm, homogenizer MA102/Plus, Marconi, São Paulo, SP, Brazil) during ten minutes. The formation of sesame oil micelles at the end of the emulsification process was confirmed by optical microscopic examination of a tenfold dilution of each emulsion.

Strains viability in emulsion was tested 24h after preparation and after sixty days of storage under refrigeration (8°C). As control, the strains were resuspended in 10% NFDM and stored under refrigeration. Ten grams of each sample were serially diluted in 0.1% peptone solution plus Tween 80 and poured on MRS agar (De Man, Rogosa and Sharpe, Difco, USA). The results, expressed as cfu/g of emulsion were calculated from MRS agar after 48h incubation at 37°C in aerobic conditions.

*Lactobacillus reuteri* 2M14C was grown in MRS broth (De Man, Rogosa and Sharpe, Difco, USA) for 18h at 37°C. This activated culture was centrifuged (2000xg, 10min, 4°C) and resuspended in PBS in order to obtain 10<sup>9</sup>cfu/mL. One hundred microliters of this suspension were inoculated intragastrically into germ-free mice. The control group received PBS. The success of monoassociation was checked by investigating the number of cfu/gram of feces during ten days.

Peritoneal macrophages from monoassociated and germ-free mice were obtained as follows: the peritoneal cavity was washed with approximately eight ml of RPMI 1640 medium (Sigma Chemicals Co., St. Louis, MO, USA); this

suspension was centrifuged (1000xg, 10min), and the recovered cells were resuspended in medium RPMI 1640 at a final concentration of 1x10<sup>5</sup>cells/mL. The phagocytosis test was done as described by Giaimis *et al.* (1992). Briefly, peritoneal cells were plated in 24-well plates with a round glass slide at the bottom for 2h at 37°C in a 5% CO<sub>2</sub> incubator. After this time, the medium was removed and 100µL of an autoclaved *Saccharomyces boulardii* suspension containing 10<sup>8</sup>cfu/ml was added to each well following incubation for 1h at 37°C in a 5% CO<sub>2</sub> incubator. The glass slides were then covered with 1% tannic acid before staining with May-Grumwald-Giemsa. Two hundred cells per slide were evaluated and attached yeast (violet) and internalized yeasts (pink) were enumerated separately. The results were expressed as number of attached and internalized yeast per macrophage.

The data were statistically analyzed using the Student's *t*-test at a probability level of 0.05. Statistical analyses were performed using the Sigma Stat program (Jandel Scientific Software, version 1.0, San Rafael, CA, USA).

## RESULTS AND DISCUSSION

Sesame oil heated at 70°C before mixing with other emulsion components generated spherical oil bodies with a diameter sufficient to entrap lactobacillus cells (Figure 1). Sesame oil must be heated at 70°C before mixing with other emulsion components in order to generate spherical oil bodies with a diameter sufficient to entrap lactobacillus cells, in accordance with results by Hou *et al.* (2003). Heating sesame oil at 40°C before mixing was unable to generate micelles of sufficient size to cover lactobacilli cells and the viability of one strain tested (*L. reuteri* 2M14C) in emulsion prepared with sesame oil heated at 70°C was higher than in an emulsion of sesame oil heated at 40°C (data not shown). Both sesame and sunflower oil are food grade ingredients and have been utilized as emulsion components and other formulation systems to carry genes, antigenic proteins, drugs and microorganisms (Sriamornsak *et al.*, 2004; Nam *et al.*, 2009; Ying *et al.*, 2010).

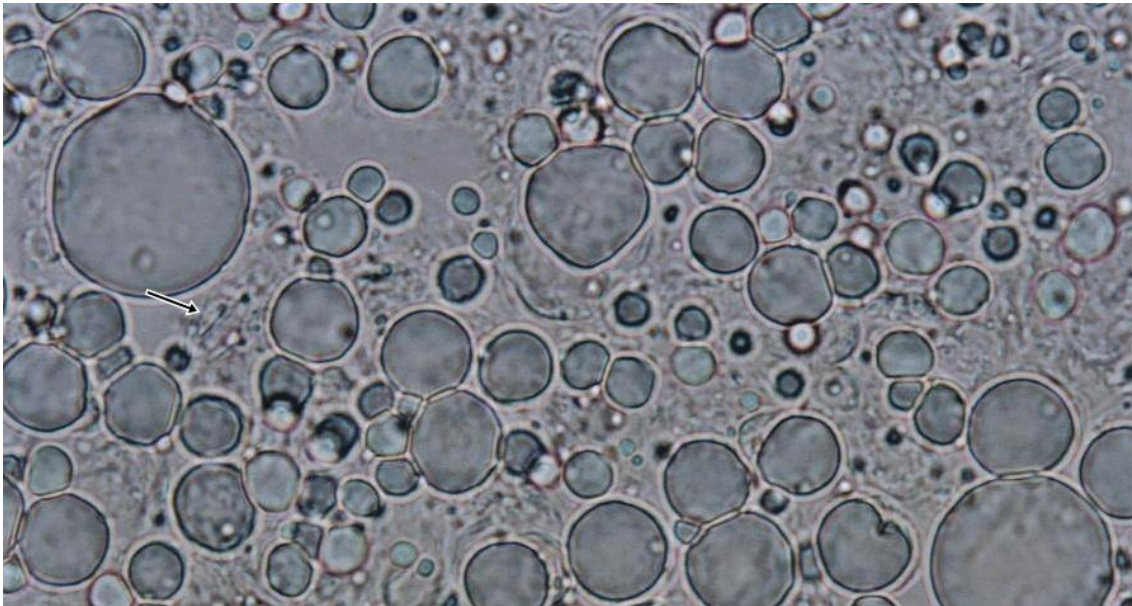


Figure 1. Light microscopy of W/O sesame oil emulsion containing *Lactobacillus reuteri* 2M14C cells. Arrow shows a lactobacillus cell. Original magnification: 100 X.

The survival of all lactobacilli strains in the emulsion at the first day was similar to the survival in 10% NFDM, used as control (Figure 2). After sixty days of emulsion storage under refrigeration, the viability of cells maintained in emulsion was higher than in control, except for *L. salivarius* 4C14C, which survived slightly better in NFDM than in emulsion and three among five strains tested survived in number equal to or higher than  $10^6$ cfu/g. The survival of strains tested herein are similar to that observed by Hou *et al.* (2003) and Chandramouli *et al.* (2004) who observed the maintenance of lactobacillus viability in artificial sesame oil emulsion and alginate acid, respectively. Lactobacillus strains showed different degrees of sensitivity to formulation storage at refrigeration for sixty days, which could be explained by eventual metabolic and morphologic differences between them. Among the strains tested, the less sensitive was *L. reuteri* 2M14C, followed by *L. salivarius* 5C14C and *L. vaginalis* 5M14E, in this order. These strains survived in number not inferior to  $10^6$ cfu/g of emulsion at the end of storage time.

Resistance to stressing gastrointestinal conditions is very important for probiotics to perform their

beneficial effects on the host. Soon after isolation, all the strains tested herein showed high resistance to acid pH, showing low or no reduction in the number of viable cells after 3h of incubation in simulated gastric juice. Susceptibility to bile salts was variable among the five strains tested. *L. reuteri* 2M14C demonstrated less inhibition after six hours of incubation in the presence of bile salts (approximately 30%) and *L. salivarius* 5C14C was more strongly inhibited by the presence of bile salts (approximately 70%) (Mota *et al.*, 2006).

Once *L. reuteri* 2M14C demonstrated good tolerance to gastric juice and bile salts and the best survival after sixty days in the emulsion ( $10^7$ cfu/g), its resistance capacity to gastrointestinal conditions *in vivo* was evaluated. The use of the germfree model is quite appropriate to achieve this goal and it has been used for a long time in the selection of probiotics (Martins *et al.*, 2012). The high fecal level of *L. reuteri* 2M14C in monoassociated mice up to ten days (Figure 3) indicates its ability to bypass the stressing conditions of the gastrointestinal tract, one of the putative characteristics of potential probiotics (FAO/WHO, 2001).

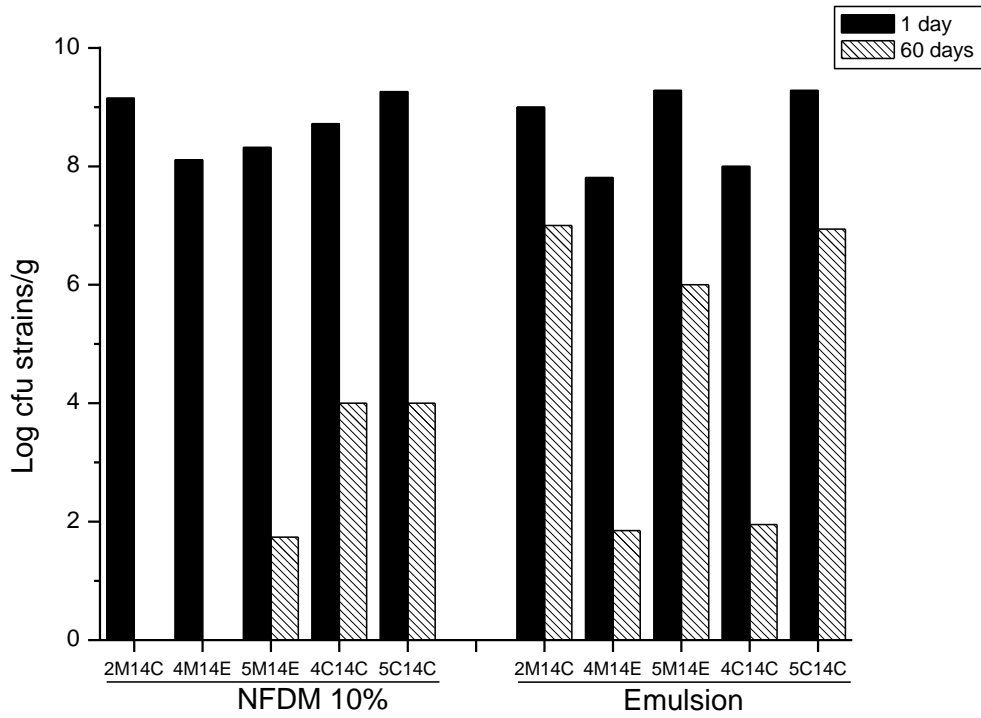


Figure 2. Survival (log cfu/g) of each *Lactobacillus* strain in emulsions and 10% NFDM (control) on the first day (black bars) and after sixty days (hatched bars) of storage under refrigeration.

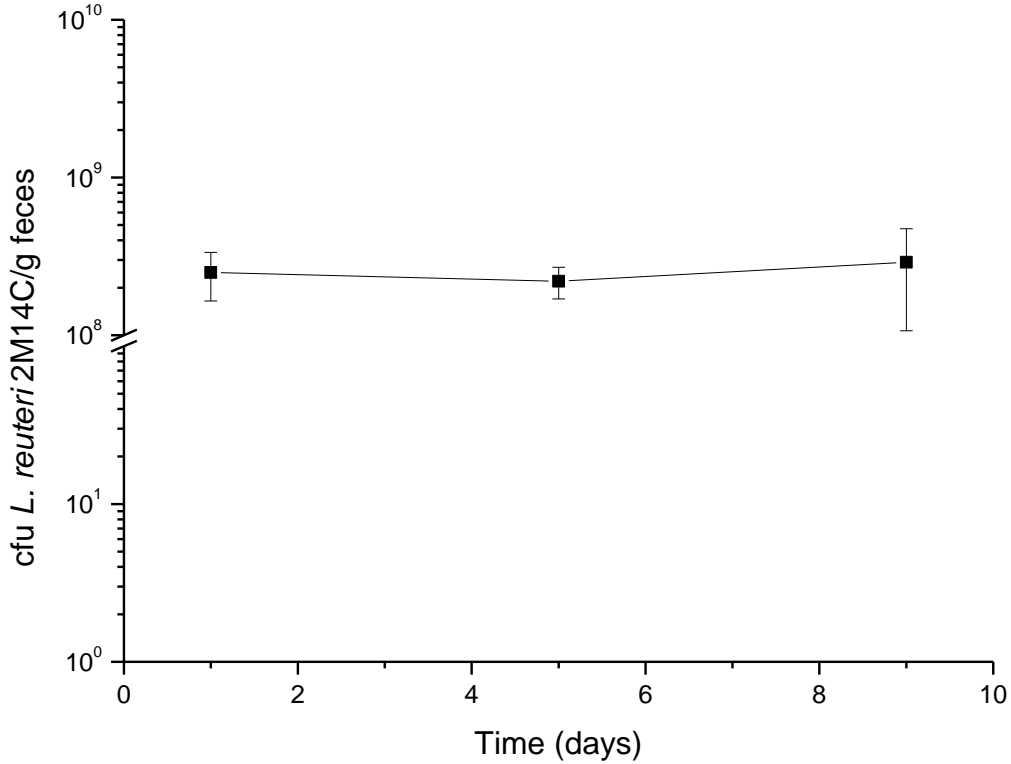


Figure 3. Number of viable *Lactobacillus reuteri* 2M14C cells in monoassociated mice feces.

We demonstrated herein that oral administration of *L. reuteri* 2M14C improved ( $P < 0.05$ ) the phagocytic capacity of peritoneal macrophages in mice, an innate immune defense mechanism against pathogens (Figure 4). This is a very important result since probiotics have previously

been associated with the activation of innate immunity through phagocytic cells and this feature is important in resistance against infections and performance improvement of broiler chicks (Higgins et al., 2007; Higgins et al., 2008; Neumann et al., 2009).

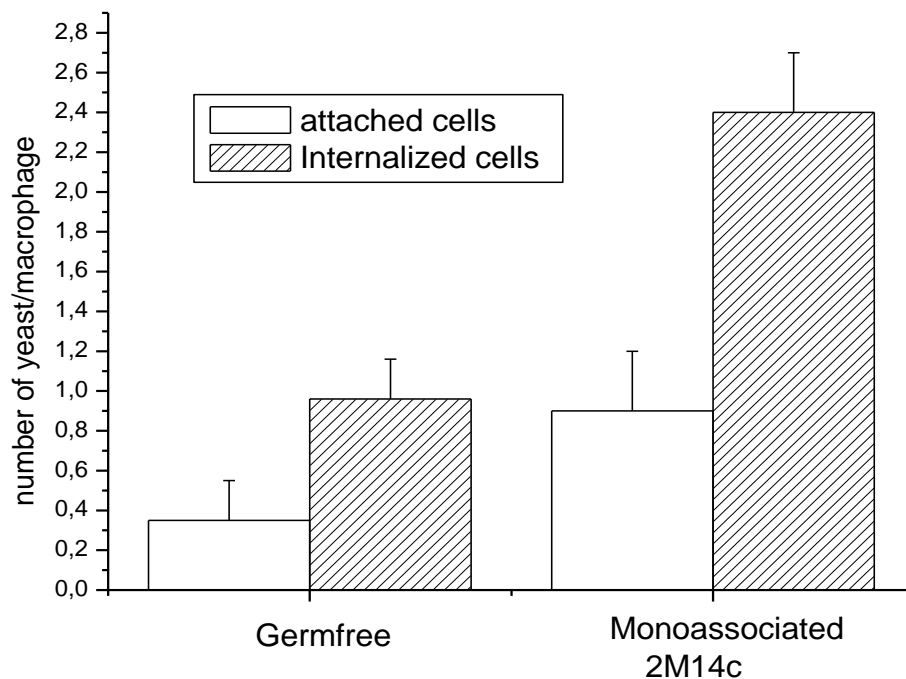


Figure 4. Number of autoclaved *Saccharomyces boulardii* attached on (white bars) and internalized by (hatched bars) peritoneal macrophages from germ-free mice and *Lactobacillus reuteri* 2M14C monoassociated mice.

### CONCLUSIONS

*Lactobacillus reuteri* 2M14C showed the best capacity to survive in emulsion, was resistant to gastric juice and bile salts, persisted in the gastrointestinal tract of monoassociated mice and improved the phagocytic capacity of peritoneal macrophages in these mice. Since this strain has these characteristics, it is suggested that it is a good candidate to be used as a probiotic for chicken, from which it was isolated. Besides, it was demonstrated that it is possible to maintain the viability of *Lactobacillus* species in a W/O emulsion containing sesame and sunflower oils. Thus, these ingredients might be used in formulations for the incorporation of lactobacilli in animal feed.

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