Revista Mexicana de Ingeniería Química

Vol. 9, No. 1 (2010) 11-16

ANTIMICROBIAL EFFECT OF *Lactobacillus casei* STRAIN SHIROTA CO-CULTIVATED WITH *Escherichia coli* UAM0403

EFECTO ANTIMICROBIANO DE *Lactobacillus casei* VARIEDAD SHIROTA CO-CULTIVADO CON *Escherichia coli* UAM0403

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Received 1 of October 2009; Accepted 22 of December 2009

Abstract

In order to assess the antimicrobial effect of *L. casei* Shirota and prebiotic effect of a galactoside (Oligomate 55), co-cultures of this probiotic bacterium and *Escherichia coli* were made in an *in vitro* fermentation with temporary and physicochemical characteristics of key regions of large bowel. Simulations of ascending colon, transverse colon and descending colon were used. With Oligomate 55 an enhancement of up to three-fold of the growth of *L. casei* Shirota was observed compared to glucose; then, our results confirmed that Oligomate 55 had a prebiotic effect on *L. casei* Shirota, stimulating its growth. In all studied regions *L. casei* Shirota had a bacteriostatic effect on *E. coli* UAM0403. Our results showed the prebiotic effect of Oligomate 55 on *L. casei* Shirota. Despite Oligomate 55 significantly stimulated the growth of *L. casei* Shirota, *E. coli* UAM0403 was able to grow with Oligomate 55 in the same amount as with glucose. These kinds of studies are important to be considered for an adequate selection of prebiotics as a critical step during synbiotic development.

Keywords: galactooligosaccharides, prebiotic; probiotic.

Resumen

Para determinar el efecto antimicrobiano de una bacteria probiótica y el efecto prebiótico del Oligomate 55, se realizaron fermentaciones *in vitro* de co-cultivos de *L. casei* Shirota y *E. coli* UAM0403, reproduciendo características temporales y fisicoquímicas de ciertas regiones del intestino grueso. Las regiones simuladas fueron colon ascendente, colon transverso y colon descendente. En todas las regiones simuladas, *L. casei* Shirota mostró un efecto bacteriostático sobre *E. coli* UAM0403. Se observó un incremento en la población de *L. casei* Shirota; la cual se triplicó al utilizar Oligomate 55. Los resultados obtenidos mostraron el efecto prebiótico del Oligomate 55 sobre *L. casei* Shirota. El azúcar prebiótico Oligomate 55 fue capaz de estimular el crecimiento de *L. casei* Shirota, sin embargo, *E. coli* UAM0403 fue capaz de crecer con esta fuente de carbono a la misma proporción que en glucosa. Por lo tanto, para prevenir la utilización de prebióticos por bacterias patógenas, se debe realizar una adecuada selección de la fuente de carbono para el desarrollo de productos simbióticos.

Palabras clave: galactooligosacáridos, prebióticos, probióticos.

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1. Introduction

Probiotics, prebiotics and synbiotics are based on the same idea: to create foodstuffs which after ingestion multiply healthy bacteria in the intestine (de Vrese and Schrezenmeir, 2008). Probiotics are living organisms that, when ingested in certain amounts, are capable of maintaining the balance of the intestinal microbiota, while prebiotics are carbohydrates that enhance the development of probiotics (Senok *et al.*, 2005).

The ability of lactic acid bacteria to inhibit the growth of pathogenic bacteria is well known. According to Hua *et al.* (2007) lactobacilli are able to compete with pathogenic bacteria when they were incubated together, but the degree of inhibition was bacterial strain depended. The inhibition produced by lactic acid bacteria may be due to the production of organic acids such as lactic, propionic and acetic (Naaber *et al.*, 2004), hydrogen peroxide, bacteriocins, bacteriocins-like substances and possibly biosurfactants which are active against certain pathogens and may be produced by different species of Lactobacillus (Millete *et al.*, 2006).

Lactobacillus casei strain Shirota, one of the most intensively studied probiotics, has been used in the production of fermented milk products for more than 70 years, and has been proven as an important probiotic with many benefits, such as the improvement of the balance of intestinal microbiota and volatile fatty acids, antitumor action, stimulation of the immune system, and antimicrobial activity (Fujimoto *et al.*, 2008).

Prebiotics are food ingredients that stimulate selectively the growth and activity of bifidobacteria and lactobacilli in the gut and thereby to promote health. Prebiotics are non-digestible oligosaccharides which reach the human colon without being hydrolyzed or absorbed in the upper part of the gastrointestinal tract (Chockchaisawasdee *et al.*, 2004). Therefore, the crucial property of prebiotics is their effect on the microbiota of large bowel (Cummings and Macfarlane, 2002). It has been postulated that prebiotics are metabolized only by beneficial microorganisms; then, they are able to alter the composition of the gut microbiota (Macfarlane *et al.*, 2006). Among the wide variety of compounds tested as prebiotics, galactooligosaccharides and fructooligosaccharides are the most studied (Crittenden and Playne, 1996; Voragen, 1998). Oligosaccharides such as Oligomate 55 are currently industrially produced to be incorporated into food products. Oligomate 55 is the trade mark of a sugar formed by the trans-galactosilation of β-galactosidase on lactose, and consists mainly of galactooligosaccharides which main component is 4'-galactosil-lactose and other components such as lactose and monosaccharides (Sar *et al.*, 2004).

The aim of this study was to evaluate the inhibitory effect of *Lactobacillus casei* Shirota in coculture with *E. coli* UAM0403 during fermentation in an *in vitro* simulation of large bowel, using the commercial prebiotic Oligomate 55 as selective agent to improve growth of probiotic bacteria.

2. Materials and methods

2.1. Bacterial strains

Lactobacillus casei strain Shirota, isolated from Yakult (Yakult, México) and *Escherichia coli* UAM 0403 was obtained from the culture collection of the Universidad Autónoma Metropolitana. *L. casei* Shirota was maintained in Skim Milk medium (Difco, Detroit, USA) and *E. coli* UAM0403 in Nutritive Agar (B. D. Bioxon, México). Cultures were stored at 4°C in their respective media.

Lactobacillus casei Shirota isolation was performed as previously described (Tharmaraj and Shah, 2003) with minor modifications. Briefly, 1 ml of sample (Yakult) was 10-fold serially diluted (10^3 to 10^7) in sterile peptone water (casein peptone al 0.1 %, pH 7.2) and stirred thoroughly. After, 100 μl of each dilution was spread on de Man Rogosa Sharpe (MRS) agar for lactic acid bacteria (Difco, Detroit, USA), MRS-NaCl (40 g/l NaCl) agar for *Lactobacillus casei* isolation. The agar plates were incubated at 37°C for 72 h. Isolated bacteria were examined microscopically for cellular morphology and Gram stain phenotype.

2.2. Culture medium

Culture medium was prepared according to Macfarlane *et al.* (1998). The medium contained (g/l): either glucose (J. T. Baker, Phillipsburg, NJ, USA) used as control, or Oligomate 55 (Yakult Pharmaceutical, Japan), 0.4; Yeast Extract (B. D. Bioxon, México), 3; Proteose-Peptone (B. D. Bioxon, México), 1; $NaHCO_3$, 0.4; NaCl , 0.08; K_2HPO_4, 0.04; KH_2PO_4, 0.04; $CaCl_2$, 0.008; $MgSO_4$-$7H_2O$, 0.008 (all salts were purchased by J. T. Baker, Phillipsburg, NJ, USA); and Tween-80, 1 ml/l (Sigma,St. Louis, MO, USA). Initial pH was adjusted according to every step of the fermentation with HCl 0.1 M.

2.3. Co-culture conditions

The co-culture growth of *E. coli* UAM0403 and *L. casei* Shirota was performed in 50 ml vessels containing 30 ml culture medium, reproducing significant physicochemical characteristics in large bowel (ascending colon, transverse colon and descending colon). Conditions were achieved by maintaining physiological temperature (37 °C), stirring (150 rpm) and residence time as occurring in humans (Macfarlane *et al.*, 1998). Vessels were incubated in an orbital shaker with temperature control (Environ Shaker, New Brunswick, New Jersey, USA) under the following conditions of initial pH and residence time: ascending colon, 5.5 and 8 h; transverse colon, 6.2 and 12 h; descending colon, 6.8 and 10 h. Each region was simulated separately and continuity was kept by transferring 1 ml inoculum from the first vessel (region) to the next in the order presented above. At the end of the cultures the pH values of each region were measured.

2.4. Microbial growth quantification

The co-cultures were carried out in 50 ml vessels containing 30 ml culture medium starting with 10^6 colony-forming units (CFU) of *L. casei* Shirota and *E. coli* UAM0403 (inoculated with 1 % v/v of an overnight culture obtained from a single colony). The control medium contained glucose and the test medium Oligomate 55 as carbon source. At the same time, control experiments for growth testing of both microorganisms in single cultures were made at same conditions. Samples were taken periodically in order to evaluate the concentration of each species during fermentation. Bacterial growth was followed by plate culture method on selective media. For *E. coli* UAM 0403 EMB Agar (Eosin Methylene Blue Agar, B. D. Bioxon, México) was used, while *L. casei* Shirota was pour-plated on Lactobacilli MRS Agar (Difco, Detroit, USA). All the dishes were incubated at 37°C from 24 to 48 hours, and UFC/mL were counted.

2.5. Statistical analysis

The fermentation experiments were carried out in duplicate. For each replication, three samples were analyzed. Student-t test ($p<0.05$) was performed using the statistics software NCSS 2000 (NCSS, LLC. Utah, USA) in order to determine if there is a significant difference between the generation time of the *E. coli* UAM0403 in presence or absence of probiotic bacterium.

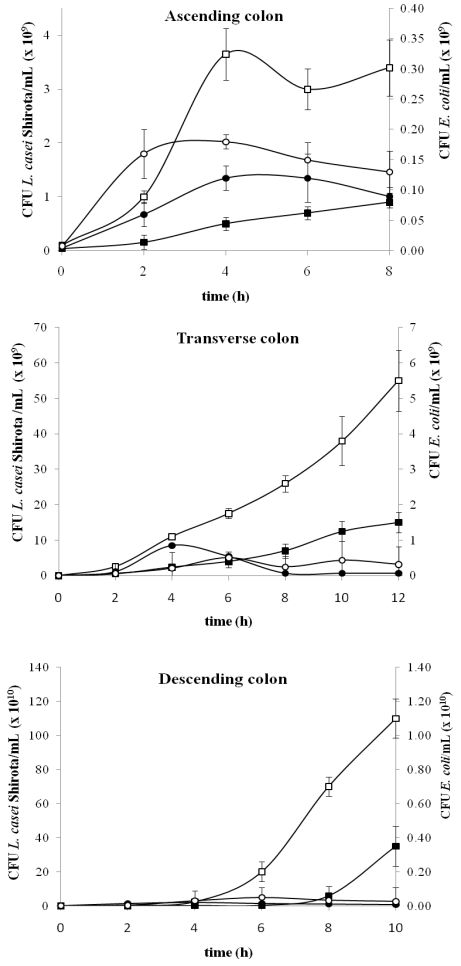

Fig. 1: Growth of *Lactobacillus casei* Shirota and *Escherichia coli* UAM0403 in co-culture. Growth curves for *L. casei* Shirota (squares) and *E. coli* (circles) with glucose (filled symbols) and Oligomate 55 (open symbols) as carbon sources. Bars correspond to the standard error of the mean ($n = 3$).

3. Results and discussion

Fig. 1 shows the growth of *L. casei* Shirota and *E. coli* UAM0403 in each studied region of large bowel. *L. casei* Shirota using both glucose and Oligomate 55 as carbon sources showed a significantly higher growth compared to *E. coli* UAM0403. The growth of *L. casei* Shirota also presented a significant increase with Oligomate 55 as carbon source compared to glucose. A maximum enhancement up to 3-fold (ascending colon, 4 h) was found.

Fig. 2 summarizes growth of both strains at the end of fermentation, growth of *L. casei* Shirota was similar in both co-culture with *E. coli* UAM0403 and single culture (control). On the oth-

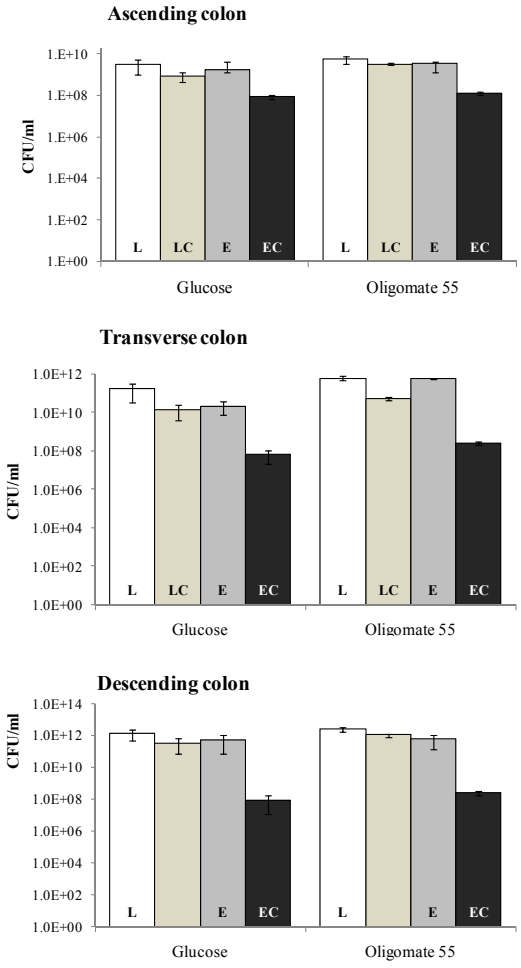

Fig. 2: Comparison between growth of *Escherichia coli* UAM0403 and *Lactobacillus casei* Shirota at final fermentation time. *Lactobacillus casei* Shirota in monoculture (L); *Escherichia coli* UAM0403 in monoculture (E). Microorganisms in co-culture was represented by LC for *L. casei* Shirota and EC for *E. coli* UAM0403.

er hand, the growth of *E. coli* decreased in presence of *L. casei* Shirota, therefore, the ability of *L. casei* Shirota to inhibit the growth of *E. coli* UAM0403 was established in these co-culture experiments. In presence of *L. casei* Shirota, growth of harmful microorganism decreased by a 4 log (descending colon, pH 6.8) compared with control. A small reduction of 2 log at transverse colon (pH 6.2) and 1 log in ascending colon (pH 5.5) in *E. coli* UAM0403 counts was also observed.

Data in the current study shown that in co-culture, *Lactobacillus casei* Shirota inhibits the growth of *E. coli* UAM0403. In contrast, growth of *Lactobacillus casei* Shirota was not influenced by presence of harmful bacterium. As shown in Fig. 1, Oligomate 55 enhanced significantly the growth of *L. casei* Shirota ($P<0.05$) compared to glucose in all studied regions. This main result agrees with Palframan *et al.* (2002); these authors observed that lactic bacteria were capable to metabolize Oligomate 55 (1 %) at pH 6 during mixed cultures with gut bacteria. Later, Huebner *et al.* (2007) studied several lactobacilli strains in batch cultures, founding a higher growth of *L. plantarum* 4008, *L. acidophilus* NCFM and *L. acidophilus* 33200 in Oligomate 55 compared to glucose.

A bacteriostatic effect of *L. casei* Shirota was observed in all regions. The growth of *E. coli* was significantly lower compared to *L. casei* Shirota; however, *E. coli* UAM0403 was able to grow in both carbon sources without significant differences in practically all regions. This can be explained by the fact that Oligomate 55 contains free monosaccharides such as glucose and galactose in more than 18 % w/w (Sar *et al.*, 2004). These results indicate that the inhibition of *E. coli* was due to the presence and metabolic activity of *L. casei* Shirota instead an effect of Oligomate 55.

In this study, was observed that the lowest pH value was reached in the ascending colon with Oligomate 55; cultures under these conditions showed the maximum pH reduction (from 5.5 to 4.1) as well as the maximum enhancement of *L. casei* Shirota growth. The minimum reduction of pH value (from 6.2 to 6.1) was found in the transverse colon with Oligomate 55, only under these conditions *E. coli* UAM0403 showed a significant growth compared to glucose. In all regions a slightly decrease in the pH value was observed, with a maximal decrease of 1.4 pH units (ascending colon with Oligomate 55). This result agrees with Fooks and Gibson (2002); who found that in 24-h cultures of *L. plantarum* 0407 and *E. coli* with fructooligosaccharides as carbon source, the pH value diminished only 1.15 units; however, inhibition of *E. coli* did occur. This suggests that besides organic acids production, other inhibitory metabolites could be produced during this kind of cultures. Brink *et al.* (2006) reported that lactic acid bacteria, such as *L. casei* LHS, produces a high level of antimicrobial activity when growing in MRS broth supplemented with glucose; they found that antimicrobial compounds with bacteriostatic effect were produced during cultures. Moreover, Millette *et al.* (2006) observed that a culture of *L. acidophilus* and *L. casei* inhibited or delayed the growth of pathogens such as *E. coli*; these authors suggested that antimicrobial activity could be due to production of organic acids and bacteriocins. Lactobacilli may exert their antibacterial activi-

Table 1. Generation time (g) of *E. coli* UAM0403 in single and co-culture with *L. casei* Shirota.

	Single culture (min)	Co-culture (min)
Glucose		
Ascending colon	28.2± 2.3^a	55.4± 2.8^b
Transverse colon	19.3± 1.7^a	54.8± 2.5^b
Descending colon	14.4± 1.1^a	48.8± 2.1^b
Oligomate 55		
Ascending colon	27.9± 2.^{a}	61.9± 3.1^b
Transverse colon	18.7± 1.8^a	39.9± 2.6^b
Descendent colon	16.2± 1.5^a	53.4± 2.8^b

^{a,b}Different letters within the same row indicate a significant difference between generation time of *E. coli* UAM0403 and in co-culture with *L. casei* Shirota ($P<0.05$).

ty through production of lactic acid and others metabolites such as hydrogen peroxide and short chain fatty acids. Also specific antibacterial compounds such as antibiotics or bacteriocins have been identified in the culture medium of several lactic acid bacteria. The mechanisms by which *L. casei* Shirota inhibited *E. coli* UAM0403 growth remain understood at present.

Generation times of *E. coli* UAM0403 are presented in Table 1. Data shown a significantly increase of bacteria generation time when grow in the presence of *L. casei* Shirota. *E. coli* UAM0403 had a generation time of 28.2 min when cultured alone in glucose media whereas the presence of the lactobacilli increased the generation time to 55.4 min. Also was observed decrease in generation time of *E. coli* UAM0403 in the simulation of the colon when it is in monoculture, indicating an adaptation of the microorganism to means. Whereas in co-culture, generation time is similar in the different regions from colon, this, suggests an effect of lactobacilli in growth of *E. coli* UAM0403. Neither, a prebiotic effect was observed in generation time of *L. casei* Shirota.

Conclusions

The results obtained indicate that the probiotic culture was able to inhibit the growth of a harmful bacterium. Moreover, results obtained in this work confirmed the effect prebiotic of Oligomate 55 during simulations of key regions of large bowel. Even though in all regions a bacteriostatic effect on *E. coli* UAM0403 was obtained, it was able to grow in both carbon sources, implying that this microorganism was capable to metabolize Oligomate 55. Further experiments must be done in order to determine which components of Oligomate 55 could be potentially consumed by other pathogens; this information may be useful in the design and optimization of synbiotics. Additional studies on antimicrobial compounds production must be done to a better understanding of the bacteriostatic effect of *L. casei* Shirota on *E. coli* UAM0403 and other microorganisms.

Acknowledgements

The authors acknowledge the financial support received from the National Council for Science and Technology (CONACyT), No. 0471-O.

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