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Effect of adding mucilage from *Opuntia ficus-indica* and *Opuntia atropes* to raw milk on mesophilic aerobic bacteria and total coliforms
Efecto de la adición de mucílago de Opuntia ficus-indica y Opuntia atropes a la leche cruda sobre bacterias mesófilas aerobias y coliformes totales

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México

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Resumen

Se evaluó la adición de mucílago líquido y deshidratado de *Opuntia ficus-indica* y *Opuntia atropes* a la leche cruda sobre el crecimiento de bacterias mesófilas aerobias y coliformes totales. Las muestras de leche cruda de vacas Holstein de un sistema de producción familiar se adicionaron con mucílago líquido o deshidratado de *O. ficus-indica* u *O. atropes* en concentraciones de 0.5, 1.0 y 2.0%. En las muestras se midió el crecimiento de bacterias mesófilas aerobias y coliformes totales. Los resultados se analizaron mediante los modelos de efectos fijos y las comparaciones entre grupos se realizaron por el método de medias de mínimo cuadrados. En general, las cuentas bacterianas para mesófilas aerobias y coliformes totales disminuyeron ($P < 0,05$) cuando se adicionó mucilago a la leche cruda al 0,5%, independientemente de la especie o de la forma física (deshidratado o líquido). Se observó disminución de cuentas bacterias para mesófilas aerobias ($P < 0.05$) cuando se adicionó mucílago deshidratado de *O. ficus indica* (Log_{10} 2.6 UFC mL^{-1}) y *O. atropes* (Log_{10} 2,7 UFC mL^{-1}) en concentración de 0.5%, respectivamente. Mientras que el grupo Control (leche cruda sin adición de mucílago) contenía Log_{10} 3.6 UFC mL^{-1} de mesófilas aerobias. La mejor respuesta para coliformes totales se observó con el mucílago deshidratado de *O. ficus-indica* (Log_{10} 3.0 UFC mL^{-1}) y mucílago líquido de *O. atropes* (Log_{10} 2,7 UFC mL^{1}), ambos adicionados al 1%. El mucílago deshidratado o líquido de *O. ficus-indica* y *O. atropes* reducen el contenido microbiano de la leche cruda.

Palabras Clave: Leche cruda; Opuntia; mucílago; bacterias aerobias mesófilas; coliformes totales

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Abstract

Raw milk is one of the most important food for human consumption, particularly for small dairy farms who produce milk either self-consumption or to sell it to local artisanal cheese makers; due to the elevate risk of contamination by pathogenic bacteria, it is important to offer alternatives to enhance microbiological quality of raw milk. Thus, the objective of this study was to evaluate the addition of liquid mucilage and dried mucilage from *Opuntia ficus-indica* and *Opuntia atropes* to the raw milk on the growth of mesophilic aerobic bacteria and total coliforms. Raw milk samples from Holstein cows supplied by a smallholder was added with liquid mucilage or dried mucilage from *O. ficus-indica* or *O. atropes* at concentrations of 0.5, 1.0 and 2.0%. Samples were measured for mesophilic aerobic bacteria and total coliforms and results were analyzed by the fixed effects model and means comparisons. In general, counts of mesophilic aerobic bacteria and total coliforms were significant reduced ($P<0.05$) when both mucilages were added at 0.5%, independently of the physical form (dried or liquid) added to the raw milk. Lower counts of mesophilic aerobic bacteria ($P<0.05$) were observed when dried mucilage from *O. ficus indica* ($\log_{10}$ 2.6 CFU mL^{-1}) and *O. atropes* ($\log_{10}$ 2.7 CFU mL^{-1}) were added at 0.5%, respectively. Control raw milk with no added mucilage had $\log_{10}$ 3.6 CFU mL^{-1}. The best response for total coliforms was observed when dried mucilage was added at 1% from *O. ficus-indica* ($\log_{10}$ 3.0 CFU mL^{-1}) and liquid mucilage at 1% from *O. atropes* ($\log_{10}$ 2.7 CFU mL^{1}). Dried mucilage or liquid mucilage from *O. ficus-indica* and *O. atropes* reduced the microbial content of raw milk.

Keywords*:* Raw milk, *Opuntia*, mucilage, mesophilic aerobic bacteria, total coliforms

Introduction

Raw cow milk is one of the most important foods for human consumption. However, high content of water, proteins, fat, carbohydrates, vitamins and minerals and its proximity to neutral pH provides the means for microorganisms to grow (Quigley *et al.* 2013, 664). In developing countries, many small dairy farms or "smallholders" produce directly the raw milk, either for self-consumption or offering it to artisanal cheese makers; therefore, it may be at elevated risk for the presence of pathogenic bacteria. Microorganisms may contaminate raw milk supplied by this way, during handling, processing and distribution after milking. This contamination could arise from the cow's udder, barn, milk collection materials, various ingredients added to dairy products (fat, water or adulterants) and dairy farm workers increasing microbial population (Garedew *et al.* 2012, 950). In addition to safety, raw milk with a higher microbiological quality allows to obtain a higher quality dairy products with an increased shelf life (D'Amico and Donnely 2010, 135). In order to propose alternatives to reduce the microbial counts in raw milk produced by smallholders it has been reported studies about the antibacterial effect of phenolic compounds on the microbiological quality of dairy products (O'Connell and Fox 1999, 523), particularly, it has been documented the inhibiting effect on growth of *Salmonella enteritidis, Staphylococcus aureus, Listeria monocytigenes* and fungi of several phenolic compounds on milk (O'Connell and Fox 2001, 113). Others studies related to the anti-microbiological effect of essencial oil of lemon, sage and thyme on dairy products were conducted by Gammariello *et al.* (2008, 4238-4146), reported a test using different types of essential oils from several citrus fruits against *Salmonella senftenberg, E. coli, S. aureus and Pseudomonas spp*. on skimmed milk in which these compound were more effective against gram positive bacteria.

Cactus pear is a group of succulent plants belonging to the *Cactaceae* family that grow principally in the arid and semiarid regions of the world, including Mexico. The most important genera of this family is *Opuntia* (Hernandez-Hernandez *et al.* 2011, 44 y 45; El-Mostafa *et al.* 2014, 14879-14901). Its rich composition in polyphenols, vitamins, polyunsaturated fatty acids and amino acids has been highlighted, many of these compounds and derivative were shown to be endowed with biologically relevant activities including anti-inflamatory, antioxidant, hypoglycemic, antimicrobial and neuroprotective properties (El-Mostafa *et al*. 2014, 14879-14901). Sánchez *et al.* (2014, 659-664) evaluated the antimicrobial and antioxidant activities of eight cultivars of cactus pear from Mexico against

Campylobacter Jejuni, *Vibrio cholera*, and *Clostridium perfringens*, they found that four cultivars were effective against *V cholera* and *C perfringens*.

Mucilage is the main complex carbohydrate compound of cladodes; this mucilage is a viscous liquid produced by the cladodes and is composed by different sugars such as rhamnose, galacturonic acids, arabinose, galactose, and xylose (Rodriguez-Gonzalez *et al.* 2014, 285) This complex polysaccharide has the capacity of absorb large amounts of water, dissolving and dispersing in self and forming viscous or gelatinous colloids (Nefzaoui *et al.* 2007, 3). According to Yahia *et al.* (2009, 625-627) and Guevara-Arauza *et al.* (2012, 1) pectic-derived oligosaccharides and mucilages of *O. ficus-indica* stimulate the growth of bifidobacteria in the colon of humans and acts a soluble receptor analogues (especially the sialic acid) for pathogens, and therefore have a direct inhibitory effect on certain pathogenic microorganisms (Magne *et al.* 2008, 585).

Ortiz-Rodriguez *et al.* (2013, 3675-3680) fed Holstein cows with diet supplemented with fresh cactus (*Opuntia ficus-indica*) and observed decreasing values of CFU mL^{-1} in both mesophiles and total coliforms in raw milk and cheese made in the artisanal way. On the other hand, Ortiz *et al.* (2011, 117) found that adding mucilage from *O. ficus-indica* or epidermis from *O. ficus-indica* or dried and milled cladodes from *O. ficus-indica* to the diet of cows significantly reduced the counts of mesophiles and total coliforms as compared to fresh raw milk.

The objective of this study was evaluate the effect of adding mucilage liquid and dried of the species of *O. ficus-indica* and *O. atropes* to raw milk on the growth of mesophiles and total coliforms.

Materials and methods

Area of study

The research was developed in the Centro Multidisciplinarios en Estudios de Biotecnología (CMEB)-UMSNH of the Facultad de Medicina Veterinaria y Zootecnia-UMSNH, located at kilometer 9.5 on the Morelia-Zinapecuaro road. 6.5 L of raw milk were supplied from a small-scale dairy herd in Cuitzillo village located at the Tarimbaro municipality, Michoacán, México. The climate in this region is warm and humid in summer with mild winter. Annual precipitation is 609.0 mm and temperatures range from 2.5 to 25.1 ºC (INEGI 2005).

Mucilage extraction

Mucilage was extracted from 4.5 kg of each species of *Opuntia ficus-indica* and *O. atropes*, with a yield of 6.2 and 12.7 g of mucilage kg^{-1}, respectively. The dried mucilage was obtained according to the methodology described by Rodríguez-González *et al.* (2014, 286, 287). Briefly, fresh cladodes were mixed in a blender for 1 min with water in a ratio of 1:8 (w/v). Then, the suspension was maintained at 83 °C for 2 h and centrifuged at 3,500 rpm for 15 min. The supernatant was added with ethanol 96% in a ratio of 1:4 (v:v). Mucilage mass was separated by centrifugation at 3,500 rpm for 15 min. Mucilage mass was dried and stored at 4°C until use. For the liquid mucilage, another methodology was proposed. Fresh cladodes were disinfected with a 2% solution of sodium hypochlorite. Cladodes were washed with distilled water. After that, cladodes were peeled, cut into slices, mixed in a blender and pressed in cheesecloth, until obtaining the mucilage in liquid form. Mucilage was stored until use in test tubes previously sterilized at 4°C.

Raw milk added with mucilage

The raw milk samples were collected from a pool of ten mature Holstein grade cows permanently confined feed with a strategy of cut and carried fresh alfalfa and supplemented with maize grain plus stover or commercial concentrated. According with the data available; the cows are mechanically milked twice per day averaging 12.5 ± 3.1 L and 3.7 ± 1.8 lactations. During the milking, the only hygienic practice utilized by the farmer was to wash the udder with warm water, the general hygienic conditions of the herd barn was considered poor.

Raw milk was collected every morning from the collecting container; previous homogenization milk was poured into sterilized flasks of 300 mL each and 16 batches of 100 mL of raw milk were obtained at the end. This procedure was repeated for 5 days, obtaining 80 samples with three replicates/samples (n=240). In each flask dried mucilage or liquid mucilage was added in the following concentrations: 0.0 (control), 0.5, 1.0 and 2.0% (w/v) with respect to the 100 mL of raw milk. The mucilage was maintained in contact with raw milk for 2 h.

Microbial enumeration

Each sample of raw milk added with dried mucilage or liquid mucilage was analyzed using the Official Norms of Mexico (SSA 1995a, 1-5; SSA 1995b, 1-13; SSA 1995c, 203-210; SSA 1995d, 1-7). One mL from each sample was transferred to a sterile tube containing 9 mL of

sterile 0.85% NaCl solution. Total coliforms were determined by the pour-plate method. Dilutions up to 10^{-6} were plated on standard plate count agar and violet red bile agar and incubated at 37ºC for 24 h. For mesophilic aerobic bacteria, samples were pour-plated in plate count agar and incubated at 37ºC for 48 h. and the bacteria counting colonies were done by using the Quebec colony digital counter.

Statistical analysis

A complete block experimental design was used in a 2x2x4 factorial arrangement with three replicates. That is, mucilage of two species of *Opuntia* was evaluated (*Ficus-indica* and *atropes*) in two forms (liquid and dried) and added to raw milk at four levels: 0.0, 0.5, 1.0 and 2.0 % based on 100 mL of raw milk. Microbial counts were changed to Log_{10} values for statistical analysis, and data were analyzed by using a fixed effects model (Littell *et al.,* 2006, 733-735). Differences between groups were obtained by the method of least square means (Littell *et al.,* 2002, 191-194). The model used was as follows:

$$Y_{ijkl} = \mu + S_i + (S*M)_{ij} + (S*C)_{ik} + (S*M*C)_{ijk} + \varepsilon_{ijkl}$$

Where:

$Y_{ijkl} = \log_{10}$ CFU mL^{-1} of the Mesophilic aerobic or Total coliforms bacteria

μ = General mean

S_i = Fixed effect of the $i_{ésimo}$ Specie with $_i$= *ficus-indica* and *atropes*

$(S*M)_{ij}$ = Fixed effect of the $i_{ésimo}$ Specie with interaction j_{esimo} form of Mucilage with $_i$= Dred and Liquid

$(S*C)_{ik}$ = Fixed effect of the $i_{ésimo}$ Specie with interaction k_{esimo} mucilage Concentration with $_i$= 0, 0.5, 1 and 2

$(S*M*C)_{ijk}$ = Fixed effect of the $i_{ésimo}$ Specie with interaction j_{esimo} form of Mucilage and k_{esimo} mucilage Concentration

ε_{ijkl} = Error (NID ~ 0, δ^2)

Results and discussion

Effect on the growth of mesophilic aerobic bacteria

Significant effects was observed in microbiological loads at different form of mucilage and levels of concentration of mucilage in both species of *Opuntia* (Table 1), however, a slight decreasing trend can be observed on microbiological count when mucilage concentration

increases (Figure 1). Effect of interactions on the growth of mesophilic aerobic was found: in the interactions Concentration of mucilage*Species of *Opuntia* (P=0.0006), Form of Mucilage*Species of *Opuntia* (P=0.0378) and Concentration of mucilage*Species of *Opuntia**Form of Mucilage (P=0.020). In general, the addition of mucilage (Figure 1), independently of the physical form added to the raw milk (dried or liquid) (Figure 2), from different species of *Opuntia* decreased significantly ($P < 0.05$) the growth of mesophilic aerobic bacteria. The effect was more pronounced when concentration of mucilage *O. ficus indica* was 1 and 2% (2.8 $\log_{10}$ CFU mL^{-1}) and 2% in *O. atropes* (2.8 $\log_{10}$ CFU mL^{-1}) (Figure 1). Although, the counts of mesophilic aerobic bacteria were not different significantly ($P > 0.05$), $\log_{10}$ 2.9 and $\log_{10}$ 3.1 CFU mL^{-1}, when mucilage from *O. atropes* was added to raw milk at concentrations of 0.5% and 1%, respectively; and, counts were different ($P < 0.05$) from the control (0% mucilage: 3.6 $\log_{10}$ CFU mL^{-1}). Low growth of aerobic mesophilic bacteria was observed when raw milk liquid mucilage *O. ficus-indica* in 1% and 2% (2.7 $\log_{10}$ and 2.6 $\log_{10}$ CFU mL^{-1}, respectively) was added and 2% of dry mucilage O. atropes (2.7 $\log_{10}$ CFU mL^{-1}). Representing a reduction of $3.4x10^{-3}$ FCU mL^{-1} aerobic mesophilic bacteria, with respect to the control (3.6 Log_{10} = $3.9x10^{-3}$ FCU mL^{-1}) when raw milk was added 2% mucilage liquid (2.7 Log_{10} = $5.0x10^{2}$ FCU mL^{-1}) or dried of *ficus-indica* or *atropes* respectively (Figure 3).

Table 1. Analysis of variance for aerobic mesophilic (Log_{10} CFU/mL) in raw milk according to the treatment

Source of variation	DF	Means of squares	F-Valor	Pr > F
Species of *Opuntia* (SO)Treatments	2	4.23578122	31.58	<.0001
Form of Mucilage (FM)*SO	2	0.44728544	3.33	0.0378
Concentration of mucilage (CM)*SO	4	0.68894489	5.14	0.0006
CM*SO*FM	4	0.40076218	2.99	0.0202
Error	182	0.13412620		
Mesophilic Mean		3.0		
Root MSE		0.36		
Coeff Var		12.1		
R-Square		0.36		

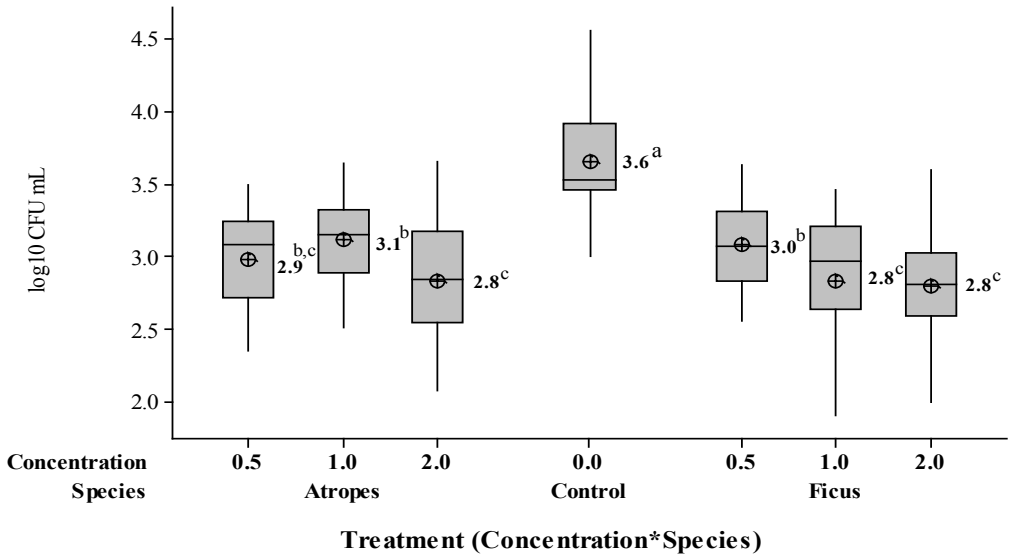

Figure 1. Least square means for mesophilic aerobic bacterias (log_{10} CFU/mL) in raw milk post addition of mucilage according to the concentration interaction*species of *Opuntia*

The decreased growth of mesophilic bacteria by adding of mucilage was also reported by Ortiz-Rodriguez *et al.* (2013, 3677-3679) when mucilage from *O. ficus-indica* was added to the raw milk (log_{10} 6.1 CFU mL^{-1}) as compared to the control with no adding mucilage (log_{10} 7.3 CFU mL^{-1}). Evidence exists that species of *Opuntia* has antibacterial properties, attributed mainly to the presence of pectic-derived oligosaccharides as mentioned by Guevara-Arauza et *al.* (2012, 997), which may explain the reduction of mesophilic aerobic bacteria and total coliforms when mucilage from *Opuntia* was added to raw milk. In our study, the values obtained of mesophilic aerobic bacteria are lower than that established by the Mexican Official Standard as safe (COFOCALEC 2004, 8). The Mexican Official Standard establishes that values of mesophilic aerobic bacteria in raw milk must be lower than log_{10} 6.1 CFU mL^{-1}.

Effect on the growth of total coliforms

In the case of growth of total coliform bacteria was only affected by the interaction of Form of Mucilage*Species of *Opuntia* (P = 0.0225), while interactions Concentration of mucilage*Species of *Opuntia* and Concentration of mucilage*Species of *Opuntia*Form of Mucilage were not significant (P = 0.0702 and P = 0.7342, respectively).

Counts of total coliforms when different Form of mucilage (dried or liquid) were added to raw milk ranged from 3.2 to Log_{10} 2.9 CFU mL^{-1}) and were significantly lower (P < 0.05) than control (3.8 Log_{10} CFU mL^{-1}) (Figure 5). However, the treatment of dried mucilage of *O.*

ficus-indica observed higher coliform counts (3.2 Log_{10} CFU mL^{-1}; $P < 0.05$) in comparison to mucilage liquid *O. ficus-indica* and dried mucilage and liquid of *O. atropes* (2.9 Log_{10} CFU mL^{-1}) (Figure 5).

Although, when triple interaction (Concentration of mucilage*Species of *Opuntia**Form of Mucilage) was not significant ($P > 0.05$) on total coliforms counts, it can be observed when mucilage dried of *O. ficus-indica* is added, less variability is observed considering total coliforms (2.8 log_{10}) in comparison to the rest of treatments (Figure 6). Finally the reduction of total coliforms in such treatment was $5.6x10^3$ UFC mL^{-1} with respect to Control, then, if UFC Log_{10} values were transformed that means $6.3x10^2$ CFU mL^{-1} (2.8 Log_{10}) of total coliforms in raw milk added with dried mucilage of *O. ficus-indica*, in comparison to $6.3x10^3$ CFU mL^{-1} (3.8 Log_{10}) of total coliforms of Control treatment (Figure 6).

The results of total coliforms observed in our study are higher than those found by Ortiz-Rodriguez *et al.* (2013, 3678), who reported lower counts of total coliforms in raw milk from cows fed with *Opuntia* used as supplement of the diet. Few studies have reported the antimicrobial activity of extracts of *Opuntia ficus-indica*; Shafiei *et al.* (2013, 907-910) studied the antimicrobial effect of methanol extracts of O. ficus-indica fruits, they found an inhibitory effect on *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Rabhi *et al*. (2013, 5875-5885) when evaluating the antimicrobial activity of extracts of O. ficus-indica they found a strong inhibiting activity against *Enterococcus facium* and *Candida albicans* growth using methanolic extracts and associated such effect to phenolic compounds. Ennouri *et al.* (2014, 1-7) evaluated the antibacterial activity of hexane extracts of *Opuntia ficus-indica* flowers at full and post-flowering stages against Gram positive (*S. aureous* and *B. subtibilis*) and Gram negative (*P. aeruginosa* and *E. coli*), they found an activity against Gram negative growth associated to camphor and aromatic compounds content.

In general, our results for total coliforms (log_{10} 3.8 CFU mL^{-1}) were lower than that found by Ortiz-Rodriguez *et al.* (2013, 3677), who reported initial counts of total coliforms of log_{10} 6.7 CFU mL^{-1} when they evaluated the effect of adding nopal cactus fractions (mucilage, epidermis and ground nopal) on aerobic mesophilic bacteria and coliform growth in raw milk. However, these authors observed similar results when raw milk was added with mucilage from *O. ficus-indica* at concentrations of 0.5%, 1% and 2%, obtaining values from log_{10} 6.1 to log_{10} 6.0 CFU mL^{-1}. It is possible that the difference in values found in this study as compared with our results was due to the different sanitary and management of raw milk; however, the effect shown was the same.

Sánchez *et al.* (2014, 659) reported antimicrobial effect of extracts of different species of *Opuntia* on *Campilobacter jejuni*, *Vibrio cholera* and *Clostridium perfringens*, in which these authors associated the bactericide activity with the phenolic and flavonoid compounds found in solvent extracts from *Opuntia*. Also, Kim *et al.* (2002, 71-78) found inhibition on the activity of *Salmonella spp.* and *E. coli* from extracts of *O. ficus-indica* var. saboten, in studies done *in vitro*. In studies *in vivo* testing extracts of O. *ficus-indica* var. saboten, not observed the presence of *Salmonella typhimurium* DT104 in feces and in the intestinal tract of mice previously challenged with the microorganism. According to the Mexican Official Standard (COFOCALEC 2005, 8), raw milk is considered safe when having a value of total coliforms lower than $\log_{10}$ 2.0 CFU mL^{-1}. The values found in this research were higher than those established by the NOM. However, we can observe that mucilage from *O. ficus-indica* and *O. atropes* promotes the reduction of total coliforms.

Conclusions

Our results showed the antimicrobial activity of *Opuntia ficus-indica* mucilage, although, such effect it has been reported in previous studies, mainly on Gram Negative bacteria, there are few reports of using mucilage on raw milk. Nevertheless it is needed to carry out further research, related to the antimicrobial effect during the time and to identity the active compound in order to develop further assays in which it can been determined appropriated minimal bacterial concentration and thus be able to offer an alternative to improve the microbiological quality enough to reduce the counts at the level established by the Official Norms in Mexico.

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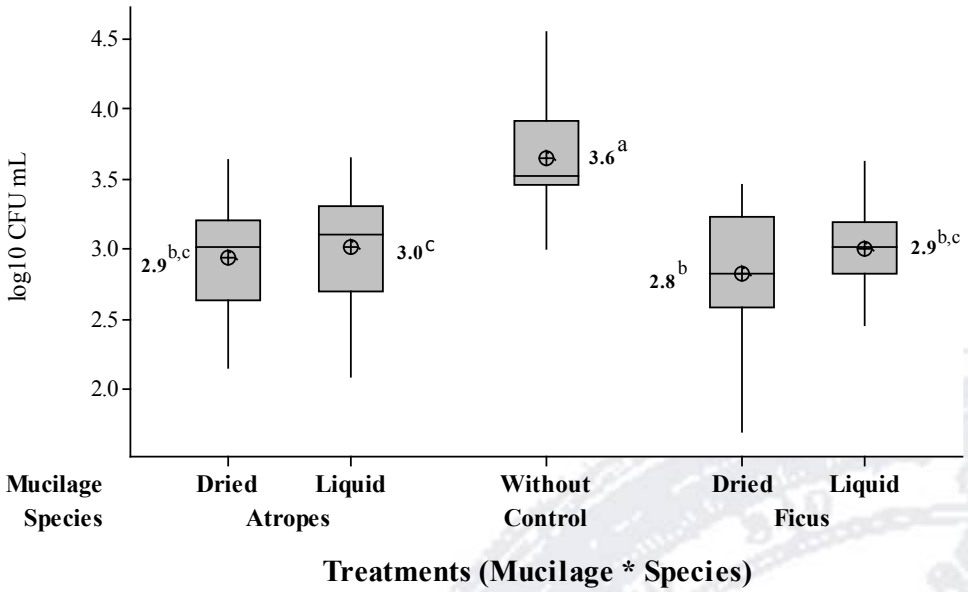
Anexos

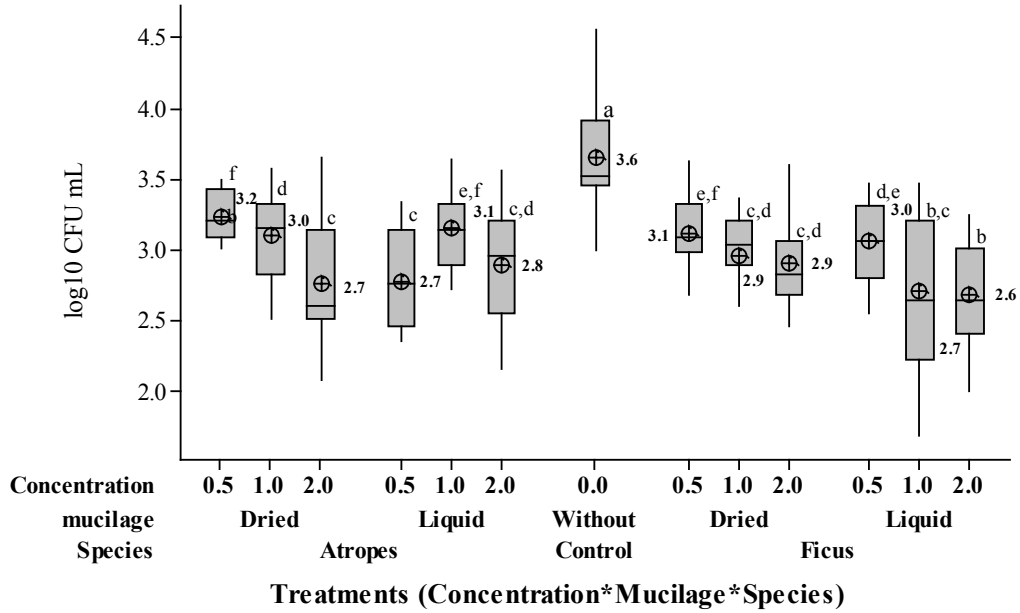
Table 2. Analysis of variance for Total Coliforms (Log_{10} CFU/mL) in raw milk according to the treatment

Source of variation	DF	Means of squares	F-Valor	Pr > F
Species of *Opuntia* (SO)Treatments	2	6.32162168	41.38	<.0001
Form of Mucilage (FM)*SO	2	0.59179423	3.87	0.0225
Concentration of mucilage (CM)*SO	4	0.33685833	2.20	0.0702
CM*SO*FM	4	0.07671983	0.50	0.7342
Error	182	0.15278341		
Coliform Mean		3.10		
Root MSE		0.39		
Coeff Var		12.6		
R-Square		0.35		



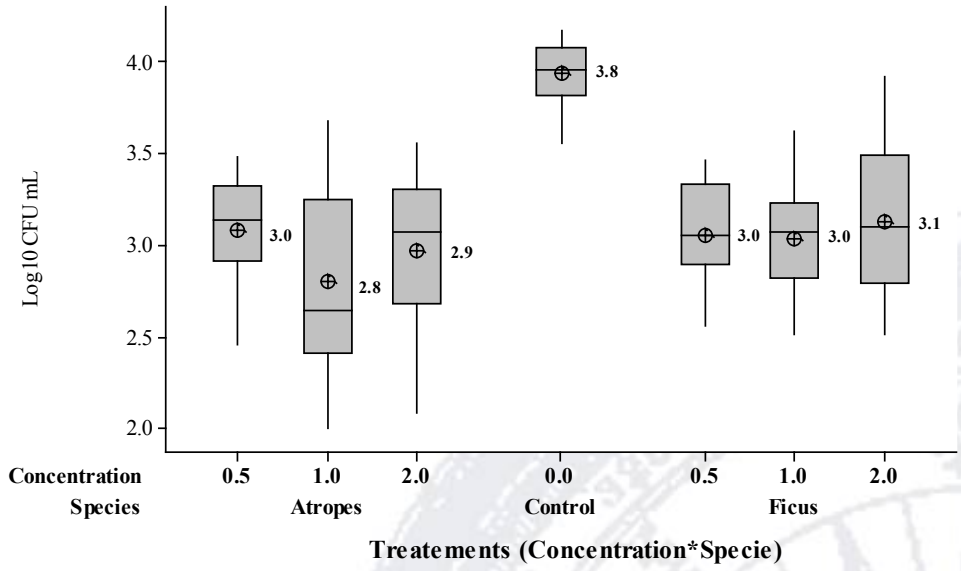
Literals a, b, c= differences between treatments (P<0.05)

Figure 2. Least square means for mesophilic aerobic bacterias (log_{10} CFU/mL) in raw milk post addition of mucilage with respect to the mucilage interaction*_Opuntia_ species



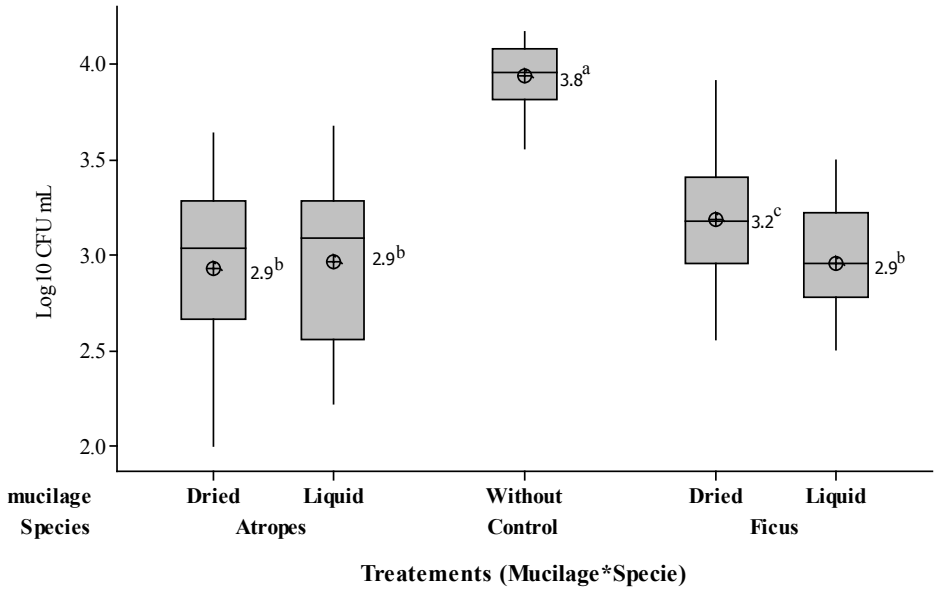
Literals a, b, c, d, f = differences between treatments (P<0.05)

Figure 3. Least square means for mesophilic aerobic bacterias (log_{10} CFU/mL) in raw milk post addition of mucilage with respect to the concentration interaction mucilage**Opuntia* species



Literals a, b, c = differences between treatements (P<0.05)

Figure 4. Least square means for total coliforms bacterias (log_{10} CFU/mL) in raw milk post addition of mucilage according to the concentration interaction*species of *Opuntia*



Leterals a, b, c = differences between treatments (P<0.05)

Figure 5. Least square means for total coliforms bacterias (log_{10} CFU/mL) in raw milk post addition of mucilage with respect to the mucilage interaction**Opuntia* species

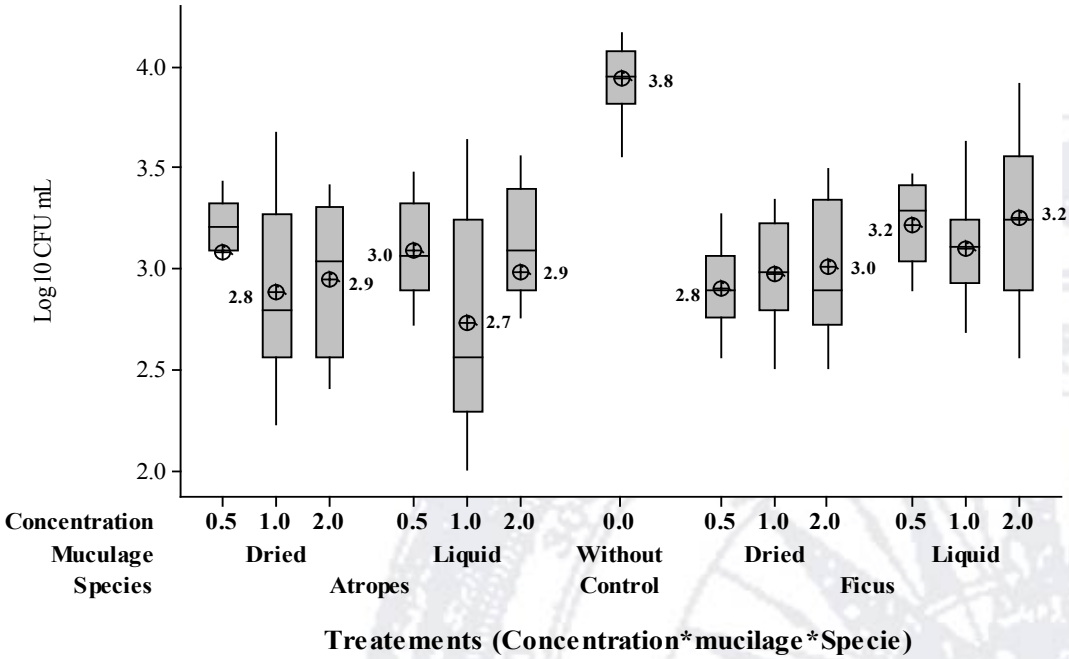


Figure 6. Least square means for total coliforms bacterias (log_{10} CFU/mL) in raw milk post addition of mucilage with respect to the concentration interaction mucilage**Opuntia* species