

Viability of *Lactobacillus acidophilus* La-5 in the Presence of *Williopsis saturnus* var. *suaveolens*

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Abstract: Maintaining the viability of probiotic microorganisms from production to consumption has long been a technological challenge for the food industry. The objectives of this study were to evaluate the *in vitro* interaction between *Lactobacillus acidophilus* La-5 and *Williopsis saturnus* var. *suaveolens* and the effect of this yeast on acidification kinetics, viability of *Lactobacillus acidophilus* and post-acidification in fermented milk during refrigerated storage at 5 °C. The *in vitro* study showed a positive interaction between the acid cell free-supernatant (CFS) of probiotic bacteria La-5 and the yeast. The addition of *W. saturnus* var. *suaveolens* increased the fermentation time due to consumption of the organic acids produced by *L. acidophilus*. During the refrigerated storage of the samples, the presence of the yeast increased the viability of *L. acidophilus* and reduced post-acidification. However, the mechanism of such interaction of bacteria and yeast is not fully understood.

Key words: Probiotic, lactic acid bacteria, yeast, *W. saturnus* var. *suaveolens*, fermented dairy products, viability.

1. Introduction

Lactic acid bacteria (LAB) have been used worldwide for thousands of years in the manufacturing of fermented foods, such as cheeses, milk, meats and vegetables [1]. In addition to traditional fermentation, LAB has also been used extensively for the development of probiotic foods. Probiotics are live microorganisms that, when administered in adequate amounts, provide a health benefit for the host [2], and their consumption has many therapeutic effects, such as the relief of lactose intolerance, action against enteric pathogens, the control and prevention of allergies, anti-carcinogenic and anti-mutagenic effects, hypocholesterolemic action and an increase in the immune response [3].

In order to exert their functional properties, probiotics need to be delivered to the desired sites in an active and viable form. However, no general agreement

has been reached on the recommended levels, and suggested levels ranged from 10^6 CFU mL⁻¹ to over 10^7 and 10^8 CFU mL⁻¹ [4].

One of the most popular probiotic foods is fermented milk [5]. However, several studies have reported the loss of viability of these microorganisms in fermented milk during production and storage. Among the factors affecting the viability of such bacteria are the solids content of the milk [6], the type of food matrix [7], the presence of preservatives and other microorganisms [8], nutrient availability [9], sugar concentration (osmotic pressure), inoculum concentration [10], incubation temperature [11], amounts of acid and hydrogen peroxide produced by the yogurt culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) [12], an increase in acidity and temperature during storage [13], concentration of oxygen in the product and oxygen permeability of the package [14].

The addition of nutrients and prebiotics [15, 16], microencapsulation [17], the use of packages that are

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impermeable to oxygen [18], and recently, co-inoculation with yeasts [19, 20] have been suggested for the improvement of probiotic viability in food systems.

Yeasts may be present in the food due to the process of deterioration, which causes undesirable changes to the products. On the other hand, many species of yeast have been safely employed for many years in the fermentation of foods and beverages. In dairy products, yeasts can interact with other microorganisms to contribute positively to the fermentation and ripening processes, a phenomenon which increases the metabolic activity of the starter culture. However, the use of yeasts in fermented milk has some limitations. The yeast employed can not ferment galactose and lactose, and no fermentable sugars should be added to the products in order to avoid the development of yeasts [19, 21].

Some researchers have shown that yeasts are capable of increasing LAB viability. Liu and Tsao [21] found that *Saccharomyces bayanus*, *Williopsis saturnus* var. *saturnus*, *Yarrowia lipolytica*, *Kluyveromyces marxianus* and *Candida kefir* increased the viability of *Lactobacillus delbrueckii* subsp. *bulgaricus* in a mixed culture of yogurt and *W. saturnus* var. *saturnus* increased the survival of *L. acidophilus*, *L. rhamnosus* and *L. reuteri*, but the same yeast failed to improve the survival of *Lactobacillus johnsonii*, *S. thermophilus* or *L. bulgaricus* in fermented milk.

Additionally, some yeast possess antagonistic properties (killer activity) toward spoilage molds and other yeasts and bacteria that is revealed by their production of killer toxins (also known as mycocins). The species *W. saturnus* var. *saturnus* and *W. saturnus* var. *mraki* are well known killer toxin producers. In cheese, *W. saturnus* var. *saturnus* inhibited the growth of *Saccharomyces cerevisiae* VL1 and *Kluyveromyces marxianus* ATCC8640. In contrast, the two spoilage yeasts grew without the killer yeast, results which indicated that *W. saturnus* var. *saturnus* could be an effective biopreservative for cheese spoilage control [22].

W. saturnus var. *saturnus* B9043 inhibited the growth of *Candida kefir*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* and *S. bayanus* in plain yogurt. The yeast also inhibited the growth of dairy molds, including *Byssoschlamys*, *Eurotium* and *Penicillium*. In addition to mycocin production, there may be other mechanisms for the antifungal activity of the yeast *W. saturnus* var. *saturnus* B9043. According to the literature, one possibility is through the action of aroma compounds, such as aldehydes and esters [23].

Yeast can also influence the physicochemical characteristics of the products. Lourens-Hattingh and Viljoen [24] employed *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Issatchenkia orientalis* and *Yarrowia lipolytica* in yogurt production. *D. hansenii* and *Y. lipolytica* were able to prevent excessive post-acidification during storage.

Williopsis sp. are not pathogenic microorganisms. They are employed in production of wines [25], cheeses [22] and juices [26], and they produce antagonist activity on other species of yeast. The species *W. saturnus* does not ferment lactose and galactose and therefore does not produce carbon dioxide or ethanol, which may cause sensory defects in fermented milks. These factors make its incorporation into fermented milks feasible and desirable [19].

There are few papers that have studied the interactions between LAB and yeasts in dairy products. To the best of our knowledge, this is the first report describing the effect of *W. saturnus* var. *suaveolens* on the viability of *L. acidophilus* La-5 in fermented milk. Considering the importance of microbial interactions and of the viability of probiotic microorganisms, the objectives of this study were to evaluate the *in vitro* interaction between *Lactobacillus acidophilus* and *Williopsis saturnus* var. *suaveolens* and the effect of this yeast on acidification kinetics, the viability of *Lactobacillus acidophilus* and post-acidification in fermented milk during refrigerated storage at 5 °C.

2. Material and Methods

2.1 Bacteria Strain and Preparation of Cell-free Supernatant (CFS)

Pure commercial freeze-dried La-5 culture (Chr. Hansen, Valinhos, Brazil) composed of *Lactobacillus acidophilus* was aseptically suspended in 1,000 mL of whole milk that had been previously sterilized (121 °C, 1 atm 15 min⁻¹). Then, 5 mL of the suspended culture was distributed into sterile flasks, and sterile glycerol was added as a cryoprotector and kept in a freezer at -18 °C. At the time of use, each lactic culture was removed from the freezer and kept at room temperature in order to be thawed out.

For use in *in vitro* assays, aliquots of 1.0 mL of the thawed culture were transferred aseptically to flasks containing 9.0 mL of MRS broth with 2 % maltose, and they were incubated at 37 °C for 72 h under anaerobic conditions (Anaerobac, Probac[®], São Paulo, Brazil). After the incubation period, the aliquots were diluted up to 10⁻⁸ using sterile bacteriological peptone (0.1%). Then, 10% (10⁸ CFU mL⁻¹) of the probiotic culture was transferred to a flask containing MRS broth with 2% maltose and incubated at 37 °C for 24 h under anaerobic conditions. After the incubation period, the broth was centrifuged at 12,500 g for 10 min at 4 °C to remove the cells. The CFS was used to evaluate the *in vitro* interaction between compounds produced by La-5 and *Williopsis saturnus* var. *suaveolens* [27]. In one sample of the CFS, the pH value was not altered (acid CFS), and in the other sample, the pH value changed to 6.8 after the addition of 1 N NaOH (neutralized CFS) and was then filtrated through 0.22 µm filters (Millipore, Bedford, USA) [28].

2.2 Yeast Strain

A lyophilized culture of *Williopsis saturnus* var. *suaveolens* 6722, obtained from the collection of tropical cultures at the André Tosello Foundation (Fundação André Tosello, Campinas, Brazil), was

rehydrated, inoculated in a test tube containing 5 mL of YM broth (yeast malt extract: 1% glucose, 0.3 % malt extract and 0.5% bacteriological peptone), and incubated at 28 °C for 48 h. After the incubation period, plates containing the YM agar (1% agar) were streaked with the broth and incubated at 28 °C for 5 days. The Gram test was performed to confirm the purity of the yeast culture.

The culture was then inoculated in the YM broth and incubated at 28 °C under agitation at 200 rpm in order to establish the incubation time required for the yeast to reach 10⁴ CFU mL⁻¹ for further analysis on the *in vitro* test. The population was determined after 12, 24, 36, 48 and 120 h, at which time the pour plate technique was applied. The colonies were counted on YM agar using aerobic incubation at 28 °C for 72 h.

2.3 Effect of Temperature, pH and Protease on CFS

The growth of *Williopsis saturnus* var. *suaveolens* was verified in the presence of acid CFS and neutralized CFS of La-5. Both CFSs were filtered using a membrane filter (0.22 µm, Millipore, Bedford, USA) according to Todorov and Dicks [29].

To evaluate the effect of temperature treatment, the acid and neutralized CFSs were exposed to heating (75 °C for 30 min; 100 °C for 15 min and 121 °C for 15 min), and refrigeration at 4 °C for 2 h. The effect of pH was evaluated by adjusting the pH value of the CFS (both acid and neutralized) to 2, 4, 6 and 8 using solutions of 1 N NaOH or 1 N HCl. The CFS samples were incubated at 37 °C for 2 h; after that, the pH levels were readjusted to their previous levels. The sensitivity of the CFS to the protease produced by *Aspergillus saitoi* was also checked (Sigma Diagnostics, St. Louis, USA). The neutralized CFS was treated with 1 mg mL⁻¹ proteinase K, and it was then incubated at 30 °C for 2 h and, afterward, heated to 95-97 °C for 5 min in order to inactivate the enzyme [30]. After the treatments, the effect of the CFS on the growth of *Williopsis saturnus* var. *suaveolens* was evaluated.

2.4 *In vitro* Interaction between Compounds Produced by La-5 and *Williopsis saturnus* var. *suaveolens*

A well-diffusion assay was performed in order to evaluate the interaction between biocompounds produced by La-5 and *Williopsis saturnus* var. *suaveolens* [31]. The yeast (10^4 CFU mL⁻¹) was pour-plated on a YM agar and incubated at 28 °C for 24 h. Afterward, wells that were 5 mm in diameter were made on Petri dishes, and 100 µL of the treated CFS were added (according to item 2.3) to the wells. The plates were reincubated at 28 °C for 24 h, and after this period, zones of inhibition or positive interaction were measured in millimeters.

2.5 Fermented Milk Manufacturing

Commercial milk powder (Ninho®, Nestlé, Araçatuba, Brazil) was reconstituted in order to obtain 140 g L⁻¹ of dry matter. The amounts of fat, solids non-fat and proteins of the reconstituted milk were determined using an ultrasonic Ekomilk analyzer (Eon Trading, Bulgaria). Milk was exposed to thermal treatment at 90 °C for 10 min, and it was cooled in an ice bath to 37 °C. Then, the milk was inoculated with 0.4% of the previously prepared La-5 inoculum (10^8 CFU mL⁻¹) and with *W. saturnus* var. *suaveolens* (10^4 CFU mL⁻¹), and the two treatments were subjected to fermentation: La-5 (La) and La-5 + *W. saturnus* var. *suaveolens* (LaWs). All inoculated milks were incubated at 37 °C until a pH of 4.5 was reached. Fermentation was stopped by rapidly cooling the fermented milk samples to 20 °C in an ice and water bath, followed by approximately 2 min of a manual standardized stirring, and cooling to 5 °C. The stirred product was dispensed into 50 mL plastic cups, and stored at 5 °C until the time of analysis.

The pH was monitored continuously to evaluate lactic acid activity using a digital potentiometer with automatic registration (data logger device) (Instrutherm Ltda, São Paulo, Brazil). The acidification rate was calculated as time of variation of pH, and the maximum value ($V_{\max}$) expressed in pH, units/min,

$T_{\max}$, with $T_{\max}$ being the time (in hours) necessary to reach the maximum acidification rate, and with $T_{\text{pH } 4.5}$ being the time (in hours) necessary to reach pH 4.5 [32].

2.6 Fermented Milk Analysis

Microbiological analysis and titratable acidity were carried out after 1, 14 and 28 days of storage at 5 °C. Counting of *L. acidophilus* was carried out on a MRS-maltose agar under anaerobic incubation at 37 °C for 72 h [33]. *W. saturnus* var. *suaveolens* was counted on a YM agar, and the plates were incubated at 28 °C for 72 h under aerobic conditions (Anaerobac®, Probac, São Paulo, Brazil). Anfotericin ($2.5 \mu\text{g mL}^{-1}$) and ampicilin ($100 \mu\text{g mL}^{-1}$) (Sigma Diagnostics, St. Louis, USA) were added, respectively, to MRS-maltose and YMA plates from the LaWs treatment. The counts were performed in duplicate. Titratable acidity [34], which is expressed as lactic acid content, was carried out in triplicate and was evaluated using titration with a 0.11 N NaOH solution.

2.7 Statistical Analysis

The effect of *in vitro* interaction between CFS and yeast was assessed by the presence or absence of halos of interaction, as well as by the intensity of the halos.

To assess the effect of the treatment (La or LaWs), a *t*-test was used for paired samples. To evaluate the effect of storage time (1, 14 and 28 days) ANOVA and multiple comparison Tukey test were used, both with a significance level of 5%. Minitab 15 statistical software was used for data analysis.

3. Results and Discussion

3.1 CFS and *Williopsis saturnus* var. *suaveolens* *in vitro* Interaction

Many LAB produce organic compounds that inhibit the development of pathogen and spoilage microorganisms [35]. However, *W. saturnus* var. *suaveolens* formed halos of positive interaction with the compounds presented in the acid CFS produced by

La-5. It may have been caused by the ability of La-5 to produce organic compounds, such as lactic acid, acetic acid and citric acid, which can be assimilated by yeasts as a carbon source, allowing such a strain to develop in an acid environment [36].

The bacteria may also allow the yeast to produce sulphur. Sulphur is an important nutrient for microorganisms, which require a source of assimilable sulphur for growth. Yeast, in particular, is able to use sulphur in a variety of organic and inorganic forms via the Ehrlich pathway of L-methionine metabolism. Volatile sulphur compounds (VSCs) are often significant contributors to the flavor profile of foods and beverages, such as fermented dairy and soybean products and alcoholic beverages [37]. Tan et al. [38] investigated the strains *W. saturnus* var. *saturnus* NCYC22, *W. saturnus* var. *subsufficiens* NCYC2728, *W. saturnus* var. *suaveolens* NCYC2586, *W. saturnus* var. *sargentensis* NCYC2727 and *W. saturnus* var. *mrakii* NCYC500 for sulphur compounds production, and they found that, in general, the most common VSCs produced by the *W. saturnus* yeasts included DMDS, DMTS, methional, methionol, 3-(methylthio)-1-propene (3-MTPP) and 3-MTPA. Strain NCYC22 was able to catabolize L-methionine in order to achieve the highest production of one of the key interest compounds, 3-MTPA, due to its potential anti-mutagenic property.

The neutralized CFS, which received different treatments, also showed halos of positive interaction with yeast, but it is not possible to know for sure which bioactive compound promoted this interaction. Moreover, the MRS broth used for the growth of La-5 is a source of nutrients that may have influenced the growth and the positive interaction of the yeast. On the other hand, La-5 produced bacteriocin in presence of co-culture with the yogurt starter species *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* [39]; however, in this study, the production of bacteriocin by La-5 was not detected.

The interaction between yeast and LAB or the

organic compounds they produce depends on different mechanisms and different combinations of species. Furthermore, it is believed that, for this phenomenon to happen, the yeast must reach high cell densities and must compete and interact with other organisms or compounds presented in the medium. Therefore, this interaction is still poorly understood [19].

3.2 Fermented Milk

3.2.1 Characterization of Reconstituted Milk

The levels of protein and solids non-fat ranged from 3.50% to 3.52% and from 9.56% to 9.59%, respectively. The fat content was 4.23% for both treatments. There was no variation in the chemical composition of the reconstituted milk ($P > 0.05$).

3.2.2 Fermentation Kinetics

The fermentation times of La and LaWs treatments were 17 h and 50 min and 48 h and 20 min, respectively. There were differences in the acidification curves (Fig. 1) and also in the acidification kinetics parameters of the fermented milks.

The $T_{pH4.5}$ during the La treatment was lower than that required during the LaWs treatment, and the V_{max} was higher during the La treatment ($0.045 \text{ upH min}^{-1}$) than during the LaWs treatment ($0.015 \text{ upH min}^{-1}$). There was an interaction between La-5 and yeast at the beginning of the fermentation, but afterward, the yeast delayed the acidification of the milk. In the study developed by Oliveira et al. [40], the pH of the product inoculated with a pure culture of *L. acidophilus* reached pH 4.5 after 13.8, 8.1 and 11.2 h, when the milk was supplemented with whey, casein hydrolysate and milk proteins, respectively. During the fermentation of dairy products, interaction between LAB and yeasts can take place. LAB produces organic acids such as lactic acid and citric acid. Conversely, yeasts transform these acids into alcohol and CO_2 , thereby increasing pH, which interferes with the acidification of the milk. Probiotic cultures do not produce high concentrations of lactic acid, citric acid or galactose, so the yeast's consumption of these acids

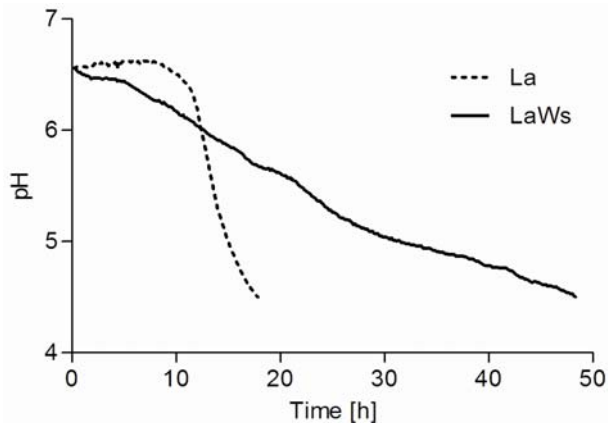


Fig. 1 Acidification curves of fermented milks.

La: fermented milk with *L. acidophilus*; LaWs: fermented milk with *L. acidophilus* + *W. saturnus* var. *suaveolens*.

causes an increase in fermentation time [41]. To prevent an increase in fermentation time, the yeast can be added after the acidification of the milk, or the probiotic can be used in co-culture with proteolytic bacteria, such as *Streptococcus thermophilus* [42].

La treatment was found to require more time to reach the $T_{\max}$ (12 h) than the LaWs treatment did (10 h 40 min), because *L. acidophilus* may have encountered greater difficulty in starting its growth in the milk in the absence of yeast (La treatment). The La treatment acidification curve reinforces this indication, since it possesses a linear region in the curve at the start of fermentation. *L. acidophilus* was found to exhibit slow growth in milk, and it is nutritionally demanding. This species requires low oxygen tension and fermentable carbohydrates, as well as proteins and amino acids, vitamins B, calcium pantothenate, folic acid, niacin and riboflavin, minerals such as magnesium, manganese iron and free fatty acids [12]. The yeasts can enhance probiotic growth because these microorganisms can metabolize the lactic acid and release vitamins such as vitamins B, pantothenic acid, niacin, biotin and riboflavin, which are important for the development of LAB [21, 26].

In another study, Oliveira et al. [15] compared, either in the presence or absence of 4 g inulin 100 g⁻¹, the results of the main kinetic parameters, specifically $V_{\max}$, $T_{\max}$, $T_{\text{pH } 5.0}$, and $t_{\text{pH } 4.5}$ in fermented milk produced

using mono- or co-cultures of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus* and *Bifidobacterium lactis* with *Streptococcus thermophilus*. In co-culture, acidification kinetics was enhanced by the addition of inulin to the milk, in such a way that it increased the maximum acidification rate and decreased the time needed to reach $V_{\max}$ and to complete the fermentation. For La mono-culture in the absence of inulin, the kinetics parameters observed were $V_{\max}$ 0.0152 upH min⁻¹, and to complete the fermentation, $T_{\text{pH } 4.5}$ was 12.9 h. These results are not similar to those obtained in the present study, because the kinetic parameters depend on the strain and food matrix used for fermentation [6].

3.2.3 Viability of *L. acidophilus* and *W. saturnus* var. *suaveolens*

The *L. acidophilus* population was higher ($P < 0.05$) in the presence of yeast over the same period of storage. There was a difference ($P < 0.05$) in the viability of the probiotic between the treatments in all of the periods under analysis (Fig. 2). There was a decrease up to 2 log cycles in *L. acidophilus* viability during refrigerated storage for La treatment, while the LaWs treatment declined up to 1 log cycle (Fig. 2).

Despite this reduction, samples of both treatments were found to have a high number of viable cells, one which would potentially be able to survive the gastrointestinal tract conditions and to produce the therapeutic effect on humans. These populations also met the requirements established by Brazilian law regarding probiotic populations, which should range from 10⁸ to 10⁹ CFU mL⁻¹ in the daily portion recommended for consumption [43].

Loss in probiotic viability was also observed in other studies, and it is one of the main problems that occur during the storage of products containing probiotics. Using the strains *L. acidophilus* La-1 e La-5 in fermented milk production, Vinderola, Bailo and Reinheimer [44] and Sodini et al. [45] reported a decrease from 2.7 to 4.6 log cycles after 4 weeks and 2 to 4.5 log cycles after 6 weeks, respectively.

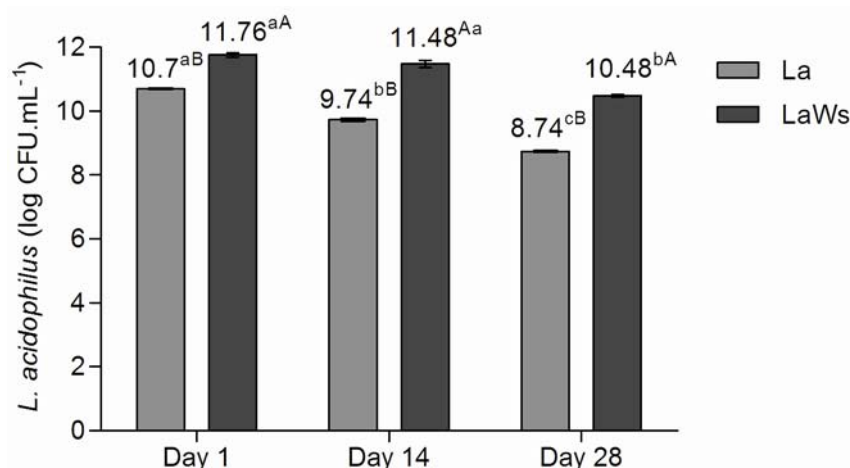


Fig. 2 Population of *L. acidophilus* (log CFU mL⁻¹) in fermented milks during refrigerated storage.

La: fermented milk with *L. acidophilus*; LaWs: fermented milk with *L. acidophilus* + *W. saturnus* var. *suaveolens*;

^{a, b}: Means followed by the same lower case letter are not significantly ($P > 0.05$) different over time;

^{A, B}: Means followed by the same upper case letter are not significantly ($P > 0.05$) different between treatments.

Variations of probiotic viability may be attributed to differences in the behavior of microorganisms and the influence of factors such as low pH after fermentation, the composition of the medium, the oxygen content in the products, fermentation and storage temperature, the low proteolytic activity of the probiotic, the availability of nutrients and the addition of inhibitory substances during the manufacturing process [12, 32, 46].

According to Viljoen et al. [41], loss of LAB viability tends to occur in the presence of yeast. However, in the present study, the addition of yeast in fermented milk had a positive effect on the cell viability of *L. acidophilus*. In a similar study, Liu and Tsao [19] observed a positive interaction between the yeast *W. saturnus* var. *saturnus* and strains of *L. rhamnosus*, *L. acidophilus* and *L. reuteri*. They found an increase in stability and cell viability of LAB when it was used in fermented milks stored at 30 °C.

Suliman and Tsenkova [47] observed that the co-inoculation of the yeast *Pichia membranefaciens* increased the population of *L. fermentum* in HTST milk after 48 h of fermentation, because the yeast produced pyruvate, amino acids and vitamins, all of which are essential for the metabolism of the bacteria.

In their non-viable form, yeast can act as source of nutrients for the development of LAB, because they

release the cell extract, vitamins and amino acids, and in doing so, contribute to an increased and/or maintained viability of LAB during shelf life of the product [19]. The interaction between the LAB and the yeast depends on the products of the metabolism of these microorganisms, the different mechanisms of action and, therefore, the different combinations of species. Furthermore, these mechanisms still need to be better understood [36, 48].

W. saturnus does not ferment lactose or galactose; thus, this microorganism is not expected to grow in fermented milk, which typically contains both sugars [19]. There was a decline in the *W. saturnus* var. *suaveolens* population during refrigerated storage of fermented milks, and on day 28, the population of this microorganism was 10^3 CFU mL⁻¹ (Fig. 3).

The growth of yeasts in dairy products occurs because of the microorganism's ability to tolerate low temperatures and low water activity and acidity, and to assimilate organic acids. They also perform lipolytic and proteolytic activities on milk casein and milk fat, and they possess the ability to resist salinity and chemicals [49].

The use of mycocinogenic yeasts for biopreservation and enhanced probiotic viability in dairy products has received little attention. However, several hurdles must

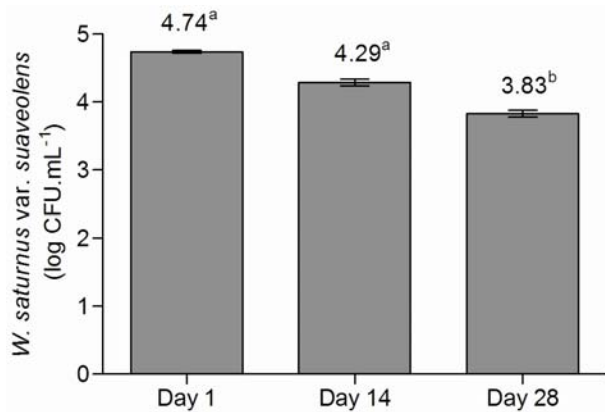


Fig. 3 Population of *W. saturnus* var. *suaveolens* (log CFU mL⁻¹) in LaWs fermented milk during refrigerated storage.

^{a, b}: Means followed by the same lower letter are not significantly ($P > 0.05$) different over time.

be overcome before yeast can be used as a biocontrol in dairy products or to maintain the stability of probiotics in fermented milks, including the high fermentation time resulting from the presence of yeast, the stability of aroma and possible deterioration [19].

3.2.4 Titratable Acidity

The levels of acidity of the fermented milks ranged from 0.86% to 1.03% of lactic acid. Fermented milks from both treatments were found to experience post-acidification during storage. Significant differences ($P < 0.05$) in the acidity of fermented milks from La and LaWs treatments were observed during refrigerated storage. Moreover, there was no statistical difference between the treatments on day 1, but a statistical difference was found between the treatments after 14 and 28 days of storage (Table 1), with higher acidity in the product without yeast.

The post-acidification of fermented milk was also reported by several other authors. The acidity of yogurts may change to a greater or lesser degree depending on the initial value of the acidity, refrigeration temperature, storage time and post-acidification activity of cultures [13]. In the present study, there was a more pronounced production of lactic acid between the 1st and 14th day in both treatments, and after this period, post-acidification continued with less intensity. The increased acidity during the first seven days of storage is related to

Table 1 Titratable acidity (% lactic acid) of fermented milks during refrigerated storage.

Treatment	Day 1	Day 14	Day 28
La	0.86 ^{ca} ± 0.01	0.98 ^{ba} ± 0.00	1.03 ^{aA} ± 0.00
LaWs	0.87 ^{ca} ± 0.00	0.94 ^{bb} ± 0.00	0.96 ^{aB} ± 0.00

Results are presented as mean ± SD.

La: Fermented milk with *L. acidophilus*; LaWs: Fermented milk with *L. acidophilus* + *W. saturnus* var. *suaveolens*;

^{a, b}Means followed by the same lower case letter are not significantly ($P > 0.05$) different over time;

^{A, B}Means followed by the same upper case letter are not significantly ($P > 0.05$) different between treatments.

consumption of lactose and production of lactic acid and galactose, results which reveal the existence of metabolic activity of LAB [50].

In studies on post-acidification of yogurt with and without the addition of *L. acidophilus*, it was found that, in the products supplemented with these bacteria, the post-acidification levels were lower than in yogurts without it. It suggests that *L. acidophilus* can reduce post-acidification problems and the subsequent release of serum during storage and products sales [10, 13]. Furthermore, *L. acidophilus* promotes less acidification in fermented milks and in yogurt during storage after processing than other LAB do [12, 51].

Viljoen et al. [41] observed a reduction in pH in yogurts over storage time at 25 °C, possibly because the yeasts tested had the ability to ferment the glucose and galactose present in the medium. However, this study found that, in yogurts stored at 5 °C, there was no significant decline in pH because the yeast took longer to initiate their development.

W. saturnus var. *suaveolens* consumed the lactic acid produced during the storage of fermented milk, a phenomenon which contributed to the lower acidity of the resulting product. Because the species *W. saturnus* does not ferment galactose or lactose, it will not metabolize sugars that release fermentation metabolites, such as lactic acid, which would result in increased acidity. This phenomenon may also have enhanced the higher viability of *L. acidophilus*, since this probiotic strain is sensitive to environments with high acidity [19].

4. Conclusions

The compounds produced by La-5 interacted positively with *W. saturnus* var. *suaveolens* and intensified its development. The addition of *W. saturnus* var. *suaveolens* had a positive effect on the population of *L. acidophilus* La-5 in fermented milk during 28 days of storage. From a practical standpoint, several hurdles must be overcome before yeast can be used to keep the population of probiotics in fermented milk stable and at high numbers.

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