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Antifungal Activity of *Lactobacillus plantarum* and Sage Extract on *Aspergillus Fumigatus* in Yogurt

Amal Bakr Shori[*], Saadyh Albloushi

Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia

Email address:
shori_7506@hotmail.com (A. B. Shor)
[*]Corresponding author

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Abstract: *Aspergillus fumigatus* is a common fungus that causes dairy products contamination. The aim of this study was to evaluate *in vitro* antifungal activity of *Lactobacillus plantarum* and sage extract on the growth of *Aspergillus fumigatus* in yogurt during 1, 7, & 14 days of storage at 4°C. Two different groups of yogurt samples were prepared. The first one consisted of the plain yogurt with *L. plantarum* + *A. fumigatus* (control), and sage yogurt with *L. plantarum* + *A. fumigatus*, and the second group contained plain yogurt + *A. fumigatus* (control), and sage yogurt + *A. fumigatus*. Both groups were investigated for pH, total titratable acidity (TTA), viable cells counts of *L. plantarum* and inhibition of *A. fumigatus* growth during refrigerated storage. The pH values of all samples were decreased non-significantly ($p>0.05$) during storage whereas TTA ranged between 1.6% - 1.7% at the end of storage. The presence of sage in yogurt increased significantly ($p<0.05$) the viability of *L. plantarum* compared to control over one week of storage. Sage alone or in a combination with *L. plantarum* showed moderate growth inhibition of *A. fumigatus* in yogurt compared to their respective controls. *L. plantarum* and sage extract could be used as antifungal activity against *A. fumigatus* in yogurt.

Keywords: *Aspergillus Fumigatus*, Yogurt, Probiotics, *Lactobacillus plantarum*, Salvia Officinalis (Sage)

1. Introduction

Fermented milk has been consumed for thousands of years. It has been known to possess several nutritional strengths and characteristics that make it a valuable contribution to the dietary patterns of human's life [1]. In addition, it has a reputation of being highly nutritious and even therapeutic. Therefore in the past few decades, researchers on fermented milk such as yogurt have increased rapidly [2].

Lactic acid bacteria (LAB) such as *Lactobacillus plantarum* is one of the most diverse species that are generally used as probiotics because of their specific healthy properties in the food [3-5]. This bacteria is widely used in the fermented food's and beverages while participating in both sensory qualities of the food and the prevention of spoilage. They have antagonistic effect against various pathogenic and toxigenic organisms [6-8].

Yogurt is one of the best-known of foods that contain probiotics. The main action of probiotics can be summarized as a reinforcement of the intestinal mucosal barrier against pathogenic microorganisms [9]. LAB in yogurt can produce several compounds these including organic acids i.e. lactic acid, acetic acid, citric acid, and hippuric acid [10]. These organic acids can inhibit the growth of fungi or lower the level of toxic metabolites produced such as mycotoxin [11].

Aspergillus fumigatus is one of the most important opportunistic pathogen producing aspergillosis. It was found frequently present in different types of food included soft cheese, freeze-dried soup, juices, regular tea and sweet biscuits [12].

Salvia officinalis (Sage) is a perennial hardy sub-shrub native to Mediterranean regions and has been widely used in the preparation of many foods and traditional medicine in the Arab world because of its flavoring and seasoning properties [13]. Sage herbs have been possess a number of biological activities for the treatment of a diverse diseases including antiseptic, antibacterial [14, 15], antioxidant [16], inflammatory [17, 18], antiviral [19, 20], antitumor [21], and anti-mycobacterial [22].

Probiotics are safe and widely used in fermented milk to increase immune response and prevent diseases. It is possible to use probiotics as bio-preservatives in yogurt to control the growth of the pathogenic fungi species associated with food spoilage thus reducing the risk of foodborne Illness, prolonged shelf-life, advanced the safety of foods, and increasing nutritional value and therapeutic properties of food. Therefore, the aim of this study was to estimate the *in vitro Lactobacillus plantarum* and *S. officinalis* (sage) effects on the growth of *Aspergillus fumigatus* in yogurt.

2. Materials and Methods

2.1. Materials and Chemicals

Pasteurized full cream milk (Al-marai, Saudi Arabia) was purchased from the supermarket. Sage leaves (*S. officinalis*) were purchased from a local store in Saudi Arabia. *Lactobacillus plantarum* (EMCC 1027), and *Aspergillus fumigatus* was obtained from Egypt microbial culture collection (EMCC), Cairo, Egypt. All agar and chemicals used in the present study were purchased from Micro master, Maharashtra, India.

2.2. Water Extraction of Sage

Sage water extract was prepared according to Xavieret al. [23] with some modification. 10 gram of sage was mixed with 90 mL of distilled H_2O. The mixture was incubated in a water bath at 100°C for 5 min before filtered to clear supernatants. Sage water extract was used freshly in the production of herbal yogurt.

2.3. Preparation of Yogurt

Yogurt was prepared according to shori and Baba, [24] with some modification. Four types of yogurt samples were prepared, namely plain yogurt with *L. plantarum* + *A. fumigatus* (control), sage yogurt with *L. plantarum* + *A. fumigatus*, plain yogurt + *A. fumigatus* (control), and sage yogurt +*A. fumigatus*. Sage yogurt with *L. plantarum* + *A. fumigatus* was prepared by mixing 85 mL of pasteurized full cream milk with 10 mL of sage water extract and 5 g of starter culture containing *Lactobacillus delbrueckii subsp. bulgaricus*, *Streptococcus thermophilus* and *L. plantarum* in the ratio of 1:1:4). Full cream milk powder (4g) was added to adjust the milk solid content. The mixture was mixed carefully and placed in water bath at 42°C. The pH was determined every 30 min using pH meter until the pH of yogurt reached 4.5. Then, the yogurt was taken out and cooled down before 1 mL of spore suspensions of *A. fumigatus* (containing a spore number of ~10^5 spores/ml) inoculated into the yogurt. Yogurt sample was kept in the refrigerator at 4°C for two weeks. Plain yogurt with *L. plantarum* + *A. fumigatus* was prepared in a similar way except adding distilled water instead of sage water extract. Plain- and sage yogurt with *A. fumigatus* were prepared using the same method above except the yogurt starter culture containing *L. delbrueckii subsp. bulgaricus* and *S. thermophilus* in the ratio of 1:1.

2.4. Determination the pH and Total Titratable Acid (TTA)

Yogurt was diluted with distilled water (1:1) [25]. The mixture was homogenized and the pH reading was taken using a digital pH meter (Mettler-Toledo 320). The total titratable acid (TTA) was determined by titration with 0.1 of NaOH. A sample of yogurt (1mL) was mixed with 9 mL distilled water and 2–3 drops of phenolphthalein [25]. Titration was carried out under continuous moving until the change of a stable pink color. TTA (%) was calculated as follows:

$$TTA\% = \text{Dilution factor } (10) \times V\ NaOH \times 0.1\ N \times 0.009 \times 100\ \%$$

where V is the volume of NaOH required to neutralize the acid and 0.009 represent the weight of lactic acid (g) neutralized by 1ml 0.1N NaOH.

2.5. Determination the Viable Cell Count of L. plantarum and the Growth Rate of A. fumigatus in Yogurt

Spread plate method was used for the bacteria counts and fungal growth. Properly diluted yogurt (1 mL) was transferred to the MRS agar (for *L. plantarum*) and PDA agar (for *A. fumigatus*) plates and mixed by lope. The MRS plates were sealed by parafilm and incubated in an inverted position at 37°C for 48 h [26] whereas PDA plates incubated at 28°C for 7 days [27]. The viable cell count of bacteria was calculated as follows: "colony forming unit (CFU)/mL= (Number of colonies formed X dilution factor of the sample)/1mL of sample".

The growth of *A. fumigatus* was recorded as follows:

(−) = no growth; (+) = low growth; (++) = medium growth; (+++) = heavy growth.

2.6. Statistical Analysis

The experiments were performed in a total of three different batches of yogurt (n=3). All assays were performed in duplicates and the results were expressed as mean± S. E. M (standard error of the mean). One way analysis of variance (ANOVA), followed by Duncan's post hoc test for mean comparison were carried out using SPSS 19.0. The criteria for statistical significance was $p<0.05$.

3. Results

3.1. Changes in the pH and TTA of Yogurt

The changes in the pH and TTA in yogurt samples were showed in Figures 1& 2. The pH values of all samples were decreased non-significantly ($p>0.05$) from 4.5 to 4.3 after 14 days of storage (Figure 1). Sage yogurt in the presence of *L. plantarum* +*A. fumigatus* showed the lowest TTA

(1.1±0.08%) among the yogurt samples at first day (Figure 2). However, prolonged storage for two weeks increased ($p<0.05$) TTA to 1.7±0.17%. Other yogurt samples showed TTA ranged from 1.5% to 1.7% during 14 days of storage.

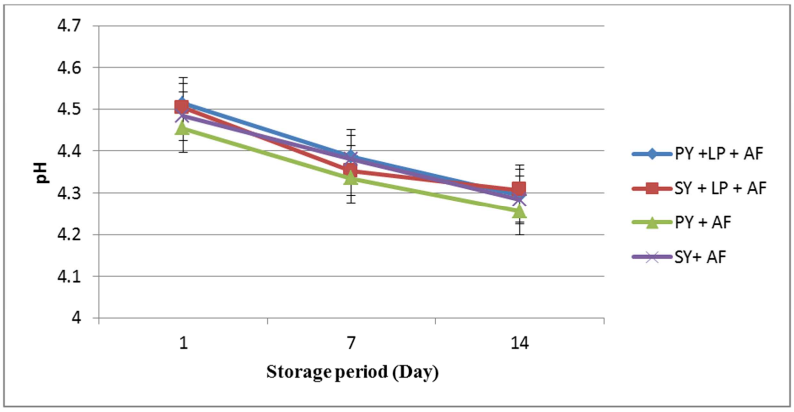

Figure 1. *Changes in pH values of yogurt samples during 14 days of refrigerated storage (4 °C). * AF= Aspergillus fumigatus, LP= Lactobacillus plantarum, SY= Sage yogurt, PY= Plain yogurt.*

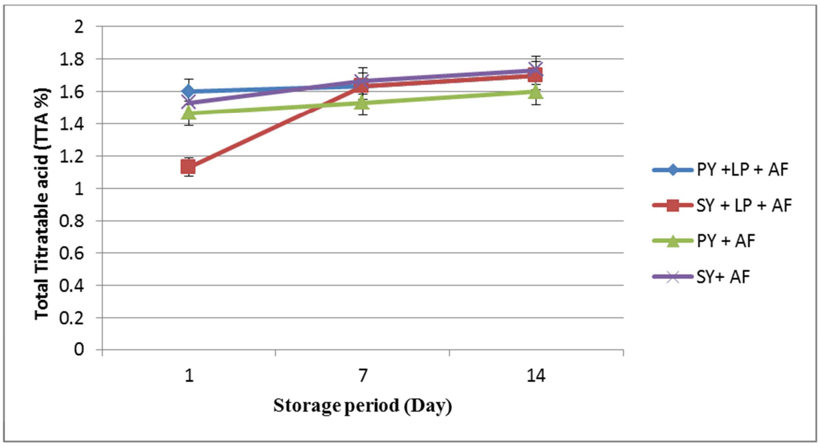

Figure 2. *Changes in total titratable acid (TTA %) of yogurt samples during 14 days of refrigerated storage (4°C). * AF= Aspergillus fumigatus, LP= Lactobacillus plantarum, SY= Sage yogurt, PY= Plain yogurt.*

3.2. The Viability of L. plantarum in Yogurt

The presence of sage extract in yogurt containing *L. plantarum +A. fumigatus* increased significantly ($p<0.05$) the viability of *L. plantarum* (0.58 and 1.17 x 10^6cfu/ml) compared to control (0.29 and 0.69 x 10^6cfu/ml) at day 1 and 7 respectively (Figure 3). However, prolonged refrigerated storage to 14 days decreased significantly ($p<0.05$) the viability of *L. plantarum* for both sage- (0.50 x 10^6cfu/ml) and plain- (0.61 x 10^6cfu/ml) yogurt.

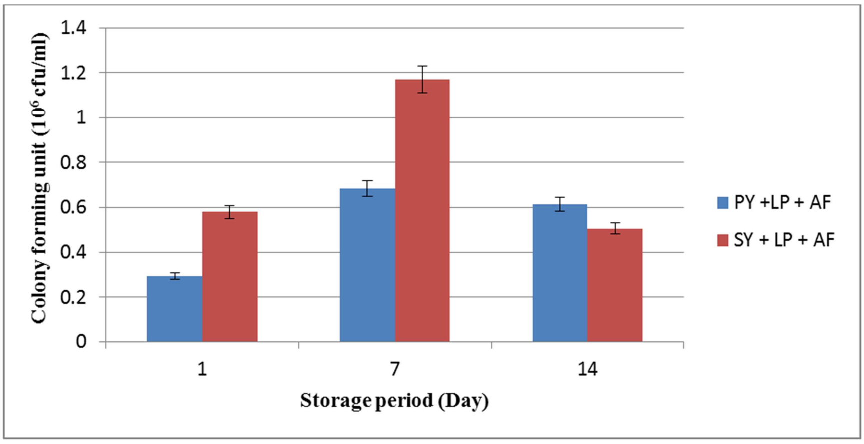

Figure 3. *The viable cell counts of L. plantarum in yogurt during 14 days of refrigerated storage (4 C). * AF= Aspergillus fumigatus, LP= Lactobacillus plantarum, SY= Sage yogurt, PY= Plain yogurt.*

3.3. Effects of Sage Extract and L. Plantarum on the Growth Rate of A. fumigatus in Yogurt

The growth rate of *A. fumigatus* in yogurt samples both in the presence and absence of sage extracts and *L. plantarum* was shown in Table 1. The addition of sage extract in yogurt either with or without *L. plantarum* showed positive effects (medium growth) on the inhibition of *A. fumigatus* compared to control (heavy growth) over 14 days of storage. Sage yogurt showed low growth of *A. fumigatus* at day 7 of storage. *L. plantarum* in yogurt had no effects on the inhibition of *A. fumigatus* growth (Table 1).

Table 1. *The growth rate of A. fumigatus in yogurt samples during 14 days of refrigerated storage (4 C).*

Storage day	*PY + AF*	*SY+ AF*	*PY +LP + AF*	*SY + LP + AF*
1	+++	++	+++	++
7	+++	+	+++	++
14	+++	++	+++	++

* AF = *Aspergillus fumigatus*; LP= *Lactobacillus plantarum*; SY = Sage yogurt; PY= Plain yogurt. (−) = no growth; (+) = low growth; (++) = medium growth; (+++) = heavy growth.

4. Discussion

Preservatives are essential to prevent food spoilage and subsequently consumers' infections by harmful pathogenic [28]. Recently, the consumers required to reduce the use of synthetic preservatives [29] thus the preserving ability of probiotic bacteria that naturally occurring in food has been increasingly employed over the recent years. Probiotics have inhibition activity against food-contaminated pathogenic microbes via the production of organic acids, enzymes, and other secondary metabolites [8, 30]. Several studies found that most fungi are sensitive to organic acid i.e. lactic, acetic and phenyllactic acids [8, 11, 30]. Lactic acid bacteria have been widely used in food as bio-preservatives which display a lot of benefits including extended shelf-life, improving food safety, and antimicrobial activity against broad-spectrum of pathogenic microbes [6].

In the present study, the decline of pH in yogurt samples over 14 days of storage occurred as a result of organic acid (i.e. lactic acid) accumulation which produced by yogurt starter culture as secondary metabolites [26]. Although the addition of sage in yogurt has significantly affected the viability of *L. plantarum* (Figure 3), however, this did not influence the post-acidification of yogurt. A similar observation has been seen by Shori and Baba [9, 31] who found there were no effects of *Allium sativum* and *Cinnamomum verum* water extracts on the yogurt post-acidification.

Sage extract presented significant effect on the viability of *L. plantarum* at first week of storage (Figure 3). This finding is in disagreement with Bachir et al., [32] who reported that *S. officinal is* extract inhibited the growth of *L. plantarum* with inhibition zone range from 2-2.5 at 10, 50 and 100 ppm concentration. On the other hand, several studies have showed that medicinal plants have positive effects on the growth of probiotics in yogurt and other dairy products [10, 24, 33-36].

Lavermicocca et al. [37] reported that some strains of *L. plantarum* have antifungal activity related to organic acids, cyclic dipeptides and low molecular mass metabolites that produced by the bacteria. Moreover, Magnusson et al. [38] have tested the antifungal activity of a large number of Lactobacillus isolates from a different environment. A number of those isolates was showed strong growth inhibition against *A. fumigatus*. *Lactobacillus delbrueckii* and *Lactobacillus rhamnosus* showed significant antifungal activity against *A. parasiticus* and *A. flavus* [39]. The present study has demonstrated moderate inhibition of *A. fumigatus* growth by sage yogurt both in the presence and absence of *L. plantarum* (Table 1). Abu-Darwish, et al. [40] observed that *S. officinalis* essential oil exhibited growth inhibition against *A. fumigatus*. The antimicrobial activity depends on their chemical composition [41]. The chemical composition of the sage essential oil has been studied [42, 43]. The major components of sage essential oils are α-thujone, 1, 8- cineole and camphor which they known for their antimicrobial activity [44, 45]. Further study is needed to evaluate and characterize the chemical composition of sage yogurt with antimicrobial components.

5. Conclusion

The presence of sage had no effect on the post-acidification of yogurt but significantly influenced the viability of *L. plantarum* during one week. In addition, sage extract alone or in a combination with *L. plantarum* contributed to preventing *A. fumigatus* contamination in yogurt. Therefore, sage yogurt with or without *L. plantarum* could have a potential effect on preventing yogurt spoilage by *A. fumigatus*. This mechanism can be applied for preventing yogurt spoilage by *A. fumigatus* during storage. Moreover, further study is needed to understand the mechanism of antifungal activity by *L. plantarum* and sage extract in yogurt and the possibility of using it as bio-preservatives.

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