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Preserving of pickled food commodities using cell free supernatants of some *Lactobacillus* and *Propionibacterium* species

Osama M. Sharaf[1]*, Sobhy M. Mohsen[2], Nadia M. AbdAlla[2], Howida M. Zaghlol[2], Lamyaa El-Sedeek[1]

[1]Food Industries and Nutrition Division, National Research Center, Dokki, Giza, Egypt.
[2]Food Technology Dept., Faculty of Agriculture, Cairo University, Giza, Egypt.

***Corres.author: sharafosama@hotmail.com**

Abstract: Large amounts of food and feed are lost every year due to spoilage by molds and yeasts. Biopreservation, i.e. the use of microorganisms as preservatives instead of chemicals, has gained increased interest. Lactic acid bacteria and Propionibacteria might be particularly useful due to their important role in many food fermentation products. The ability of cell free supernatants (metabolites) from *Lactobacillus plantarum*, *Propionibacterium thoenii* and *Propionibacterium freudenreichii* spp *shermanii* were investigated to prevent the molds and yeasts growth in manufactured pickled cucumber, green pepper, and lemon. *Lactobacillus plantarum* produced maximum amounts of antifungal substances followed by *Propionibacterium shermanii* and *Propionibacterium thoenii*.

The efficacy of *Lactobacillus plantarum* to control molds and yeasts in manufactured pickles over long storage period (60 days and 9% salt) at room temperature was evaluated.

Addition of 3% cell free supernatant of *Lactobacillus plantarum* caused a high percentage of inhibition (100%) of *Aspergillus parasiticu*s, Aspergillus *niger*, and *Penicillium* species, whereas it caused a variable in viability of wild lactic acid bacteria. It could be concluded that *Lactobacillus plantarum* have the ability to produce natural antifungal substances. These metabolites (cell free supernatant) could inhibit mould and yeast growth as will as aflatoxin production. This means that these metabolites could be utilized as natural preservatives in some foods such as pickles. In addition the inhibition of *A. parasiticus* during pickling prevented the formation of aflatoxin and consequently improved the quality & safety of pickles.

Key words: Antimicrobial, Lactobacillus, Propionibacterium, Pickled food commodities.

Introduction

For many years it has been suspected that moldy foods may cause serious chronic disease outbreaks in human and animals. Besides this problem fungal spoilage of food also causes significant economic loss in agriculture commodities as well as in food industry[1]. Worldwide, about 5–10 % of the food production is estimated to be spoiled by these organisms[2]. Fungi are significant spoilage organisms of food and feed where the potential mycotoxins produced from molds are of particular concern[3]. It has now been established that there is about 200 different types of molds form substances that are orally toxic to human or animals[4].

Mycotoxins are toxic secondary metabolites of fungal origin, when ingested inhaled or absorbed through the skin may cause lowered performance, sickness or death in human and animals. Mycotoxins have attracted worldwide attention due to the significant losses associated with their impact on human and animal health, and consequent national economic implications [5]. Aflatoxins are the most documented of all mycotoxins and have a wide products presence [6].They are a group of highly toxic secondary metabolites produced by certain strains of *Aspergillus* species in various food and agriculture commodities.

In the past decades, fungi and mycotoxins found in foodstuff might have played important roles in the carcinogenesis of lung cancer. Recently preliminary work showed that aflatoxin G_1 (AFG_1) and Sterigmatocystin (ST) detected in the liquid of pickled vegetables could induce lung adenocarcinoma in mice as well as Wistar rats[7]. Ji and Li, 1991[8] also reported that pickled vegetables cause high-risk of esophageal cancer in China, whereas Kurosawa *et al*., 2006[9] indicated that highly salted foods are important risk factors for death from stomach cancer.

Therefore there is a great demand for novel strategies to prevent both the formation of aflatoxins in food and feed and the impact of aflatoxin contamination. Biological decontamination using microorganisms is one of the well-known strategies for management of aflatoxins in food and feed. Among the different potential decontaminating microorganisms lactic acid bacteria and propionibacteria represent a unique group, which is widely used in food fermentation and preservation [10].

Lactic acid bacteria (LAB) are extensively used in the fermentation of wide variety of food products and are known for their preservative and therapeutic effects [11]. Lactic acid bacteria produce various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocins during lactic acid fermentation[12]. Moreover it has been discovered that propionic acid bacteria (PAB) can produce a antifungal material that can inhibit mould and yeast [13]. On the other hand propionibacteria gain energy through fermentation of lactate and sugars to propionate, acetate, carbon dioxide and vitamin B_{12} [14].

Recently there has been significant interest in using lactic acid bacteria and propionibacteria as a protective culture to enhance food safety and stability [15]. In addition, Tawfik et al.(2004)[16] found that addition of lyophilized *P.thonii* P-127 metabolites by 1.5% to Domiati cheese milk obviously prolonged the shelf-life of soft cheese .

Therefore the first aim of this study was to detect antimicrobial activity of metabolites produced by some *Lactobacillus* and *Propionibacterium* against fungal growth and aflatoxin production. The second aim was to evaluate the effectiveness of cell free supernatants to preventl yeast and mould growth in pickled food commodities.

Materials and Methods

Collection of samples:

Forty samples of different pickled food commodities i.e. cucumber, green pepper and lemon processed by traditional methods without pasteurization and pickled in a large barrels were collected from local markets of Cairo governorate. These pickles are sold in markets without packing and subjecting to different spoilage factors.

Cultures

Propionibacterium thoenii P-127 was supplied by Department of Food Technology Propionibacteria Culture Collection Iowa State University. *Propionibacterium freudenreichii* spp *shermanii* was obtained from Chr.Hansen's Lab., Denmark and *Lactobacillus plantarum* from Northern Regional Research Laboratory, Illinois, U. S. A. Aflatoxins B_1, B_2, G_1 and G_2 from Sigma Chemical Company, U. S. A. were used.

Isolation of fungi from pickled food commodities:

Ten grams of each sample were cutted with a sterilized knife and blended in a mortar then transferred to sterilized flask containing 90 ml of sterilized saline solution. Serial dilutions were carried out until 10^{-5}; then 1 ml was transferred on a petri dish containing Potato Dextrose agar medium. Incubation was carried out at 25°C ± 2 for 7 days [17]. Fungal colonies were counted, then picked and purified on potato dextrose agar slants

and incubated for 5 days at 25°C ± 2 ,the isolated purified fungal colonies were identified according to Nelson et al. [18].

Determination of aflatoxins in pickles :

The pickles were extracted twice with chloroform, filtered through glass wool, and then the filtrates were transferred to a separating funnel. The lower chloroform layer was passed through anhydrous sodium sulphate. The extracts were finely dried under nitrogen and stored in vials at -20°C until aflatoxin determination [10].

Spots of extracted samples and aflatoxin standards were applied on a (20 x 20 cm) pre-coated aluminum sheets of silica gel 60 without florescent indicator (TLC), activated at 105°C for 2 hours. Extracted samples were dissolved in benzene: acetonitrile (98:2 v/v). TLC plates were then developed in toluene: ethylacetate: 88% formic acid (60: 30: 10 v/v/v). After development TLC plates were dried and exposed to long wavelength ultra violet light for visual estimation [19].

HPLC equipped with a model 600 pump, and a model 474-fluorescence detector and Millennium 2010 software (Waters) was used to quantify aflatoxins. Separations were carried out at ambient temperature on Phenomenex 4µ ODS column, (250 x 4.6 mm). Aflatoxins were eluted with acetonitrile / methanol / water (1:3:6 v/v/v) as the mobile phase at a 1 ml/min flow rate. The detection wavelength for excitation and emission were set at 365 and 450 nm, respectively.

Aflatoxin concentration was calculated as ppb by the following equation according to [AOAC methods, 1990][20].

$$\text{Aflatoxin (ppb)} = (B \times Y \times S \times V) / (Z \times X \times W)$$

Whereas,

B = Area of aflatoxin peak in sample Y = Concentration of aflatoxin standard µg/ml

S = Aflatoxin standard spotted µl V = Final dilution of sample extract µl

Z = Area of aflatoxin peak in standard X = Sample extract spotted µl

W = gm sample represented by final extract

Preparation of pickles

Cell free supernatant preparation:

Lactobacillus plantarum was cultivated in MRS broth and incubated at 37°C/ 48 hrs. *Propionibacterium thoenii* were grown in sodium lctate broth and incubated at 32 ºC / 3 days. The cell free supernatant (metabolites) foe each of the tested cultures was obtained after centrifugation at 4000 rpm /m at 4 ºC for 10 minutes. The supernatants were separated from cell pellets and filtered through a 0.45 µ sterile membrane filter to remove remaining cells. Cucumber, pepper, and lemon were salted and pickled similar to those processed in the local production by traditional methods (pickling depend on natural flora of lactic acid bacteria) without pasteurization and packing according to the method described by Halabo et al.[21] and used as control. For the other treatments of pickles, metabolites were added to the brine of the pickled vegetables at concentrations 2 & 3%.

Control and treated pickles were left for 60 days at room temperature and 9% salt for pickling. Samples of pickles were analyzed at zero time and after 7, 15, 30, 45 and 60 days for mold and yeast growth as well as viability of lactic acid bacteria. Fungal growth was identified according to [18], whereas aflatoxin in pickles was also determined.

Microbiological analysis

Total Lactic acid bacteria and mould were counted on Elliker agar (37ºC/ 48 hr)and potato dextrose agar (25°C / 7 days), respectively .

Results and Discussion

Fungal Species isolated from pickled food commodities:

Data in **Table 1** indicated that per centage contamination of *A. parasiticus*, *A. niger* and *Penicillium* species were 56 ,78 , 89%; 50 , 33 , 50% and 20 , 30 , 90% in pickled cucumber , green pepper and lemon samples, respectively. It could be noticed that cucumber was highly contaminated by different fungal species, followed by green pepper and lemon respectively. It was also demonstrated that contamination by *Penicillium* species was higher in pickles samples than other fungi . The fungi isolated from pickled food (cucumber, green pepper, lemon) are common soil fungi associated with decaying vegetation and degradation of vegetables[17,22,23]. These fungi are known to gain access to vegetables during harvest and handling.

Table 1. Occurrence of fungal species in different pickling products collected from different Egyptian markets

Samples (NO.)	Pickling products	Fungal species						Total number of isolates	
		Aspergillus parasiticus		*Aspergillus niger*		*Penicillium* species			
		NPS	%	NPS	%	NPS	%	NPS	%
18	Cucumber	10	56	14	78	16	89	40	57
12	Green pepper	6	50	4	33	6	50	16	23
10	Lemon	2	20	3	30	9	90	14	20
40	Total	18	45	21	53	31	78	70	100

NPS: Number of positive samples

Aflatoxins content in pickles:

Results in **Figure 1 showed** that aflatoxin productions in pickles collected from Egyptian markets were varied according to raw material and the type of aflatoxin(B_1, G_1 and G_2). Pickled Cucumber showed highly incidence of aflatoxins B_1 and G_1 (48 and 1370 ppb, respectively). On the other hand the concentration of aflatoxin G_1 in pickled Green pepper and pickled lemon was 138 ppb and 69 ppb, respectively. Results also revealed that pickled cucumber samples were contaminated by 117 ppb aflatoxin B_2.

Similar results were reported by [7], who found that aflatoxin G_1 and Sterigmatocystin were detected in pickled vegetables, which could induce lung adeno-carcinoma in mice as well as Wister rats. It is known that maximum limits of aflatoxins production must not exceed 20 ppb in different foods [24]. However in this study, aflatoxin G1 content in the processed pickled vegetables was reached 1370 ppb. This high content of aflatoxin G1 (1370 ppb) present in vegetables cucumber collected from local market could be attributed to the unsanitary conditions used in processing. In addition the processed pickled vegetables were not heat treated (pasteurized), large barrels were used in pickling process and the pickles was subjected to different microorganisms and other contaminants.

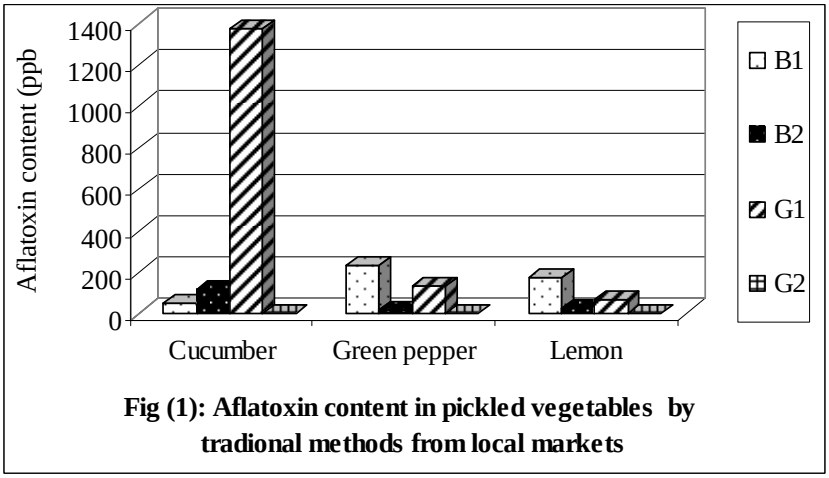


Fig (1): Aflatoxin content in pickled vegetables by tradional methods from local markets

Behaviour of fungi in pickled food commodities:

The behavior of *Aspergillus parasiticus, Aspergillus niger and Penicillium* species in the presence of cell free supernatants of *Lactobacillus plantarum, Propionibacterium thoenii* and *Propionibacterium shermanii* was studied. This behavior was changed depending on the strains used and the concentration of treatments as presented in Figures 2 - 4.

Pickled cucumber, green pepper, and lemon were manufactured using 2 and 3 % of cell free supernatants produced by *L. plantarum, P. thoenii, P. shermanii* .

Data in Figure 2 showed that 2 & 3% of cell free supernatant of *L. plantarum* completely inhibited mould and yeast growth after 7 days of storage in pickled green pepper. Wherease, 3% of cell free supernatant of *P.shermanii* was gave similar results after 45 days for mould. Among the tested supernatants, *P.thoenii* had the lowest antifungal activity against yeast. It was also noticed that counts of lactic acid bacteria increased 7 days, thereafter counts decreased during storage period for 2 & 3 % supernatants of *L. plantarum* and *P. thoenii*.

The behavior of mould and yeast in pikled lemon as affected by the addition of 2 & 3 % of cell free supernatants of *L. plantarum*, *P. thoenii* and *P. shermanii* is shoun in Figure 3 . It is clear that the addition of supernatants led to a reduced viability of mould and yeast. Cell free supernatant of *L. plantarum* had the greatest antifungal activity followed by *P. shermanii*. In addition, lactic acid bacteria in both control and pickled lemon followed the same trend, hncreased through storage period to reach the maximum counts after 15 days , thereafter counts decreased through the rest of storage .

As seen from Figure 4, the addition of cell free supernatants of *L. plantarum*, *P. thoenii* at 3 % in manufactured pickled cucumber, completely inhibited mould and yeast growth during storage period. Visual observation done on the growth of the moulds indicated that visible mycelial growth occurred in control pickles. It was also noticed that viability of lactic acid bacteria increased during storage period till the end of 15 days then the counts disappeared.

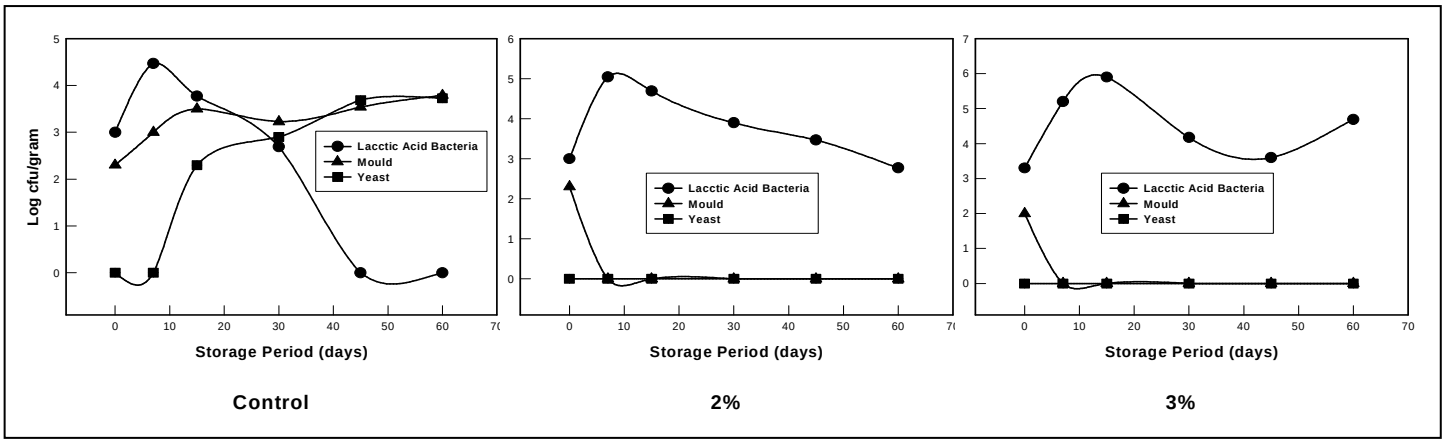
Antifungal activity has to be considered an important characteristic for selecting lactic acid and propionic acid bacteria for extending the shelf life of manufactured pickling products.

It could be indicated that on using *L. plantarum* (2 & 3%) prevented mold and yeast in green pepper, whereas in lemon and cucumber only 3% prevented mold and yeast growth. On the other hand using 3% *P. shermanii* with pickled lemon and green pepper, mold and yeast were inhibited, whereas *P. shermanii* (2 & 3%) in pickled cucumber completely inhibited both mould and yeast growth. Data also showed that *P. thoenii* (2 & 3%) did not have any antifungal activity, in pickled lemon and green pepper whereas 3% *P. thoenii* inhibited mould and yeast growth in manufactured cucumber. Thus it could be concluded that *L. plantarum* showed the highest antifungal activity and can be used in various food products. Similar results were reported that culture supernatant of *L. plantarum* has reduced fungal growth.Our results are in agreement with those obtained by [25,26,27] They reported that *L. plantarum* has previously inhibited fungal growth, preventing fungal spoil and mycotoxins forming in food and animal feed. On the other hand,Vega *et al*.,(2002)[28] found that *L. plantarum* starter culture had the potential to improve the process and provide the production of fermented green olives of consistency high quality. In addition, the results of the present study indicated that metabolites of *P. shermanii* followed by *P. thoenii* showed antifungal effect against molds and yeasts. It was suggested that it could be used as bio-preservative against molds and yeasts and increase the shelf-life [13].

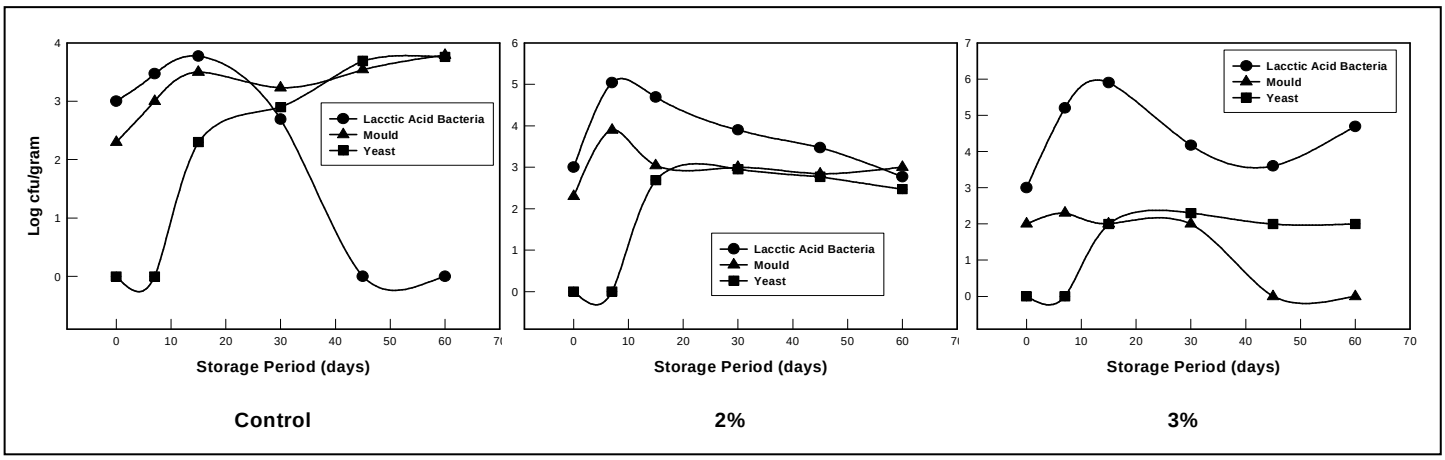
Generally, these findings are in accordance with those of AbdAlla *et al*. (2006)[10] who reported that fungal growth may be enhanced, retarded or remain unchanged as a result of growth of another microorganisms in the environment. Also, Bullerman *et al*. (1990)[29] found that the competitive growth is probably factor in the inhibition of fungal growth. On the other hand inhibition of fungal growth may be due to the antifungal activity of the natural compounds produced by lactic acid bacteria and propionic acid bacteria [30]such as organic acids (i.e. lactic and acetic acids)[31].

Aflatoxins content in processed pickled food commodities

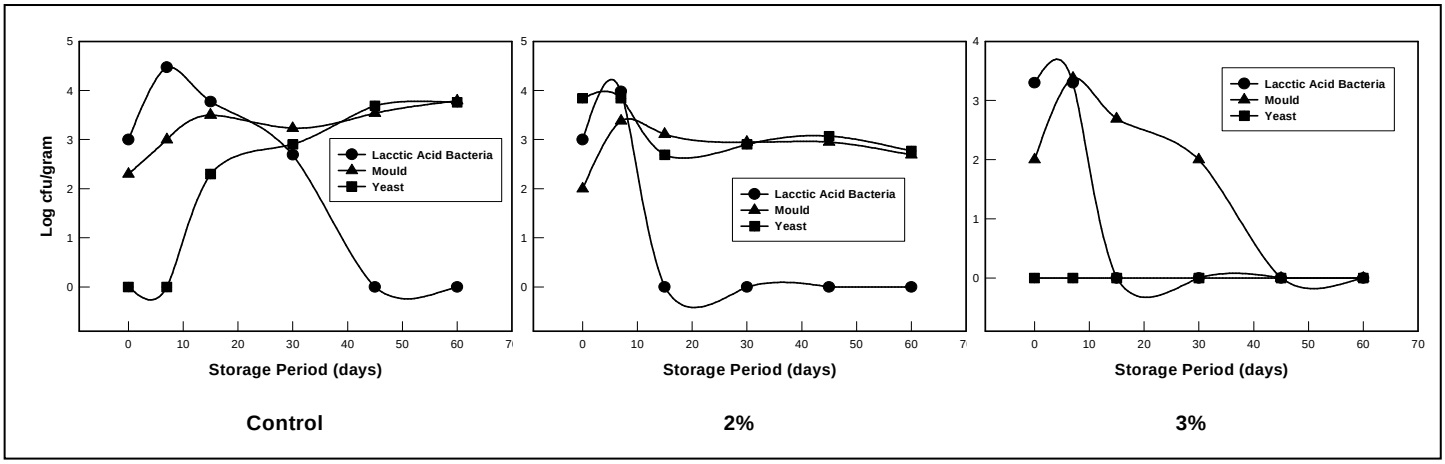
On studying aflatoxin production in control of manufactured pickles during storage,results revealed that pickled cucumber highly contained aflatoxins, the concentration of aflatoxin G_1 was 970 ppb while it was 130 and 4o ppb for pickled Green pepper and pickled lemon respectively. Data also indicated that pickled cucumber samples were contained 98 ppb for B_2 and 25 ppb for B_1(Figure 5).



Using Cell Free Supernatant of *L. plantarum*

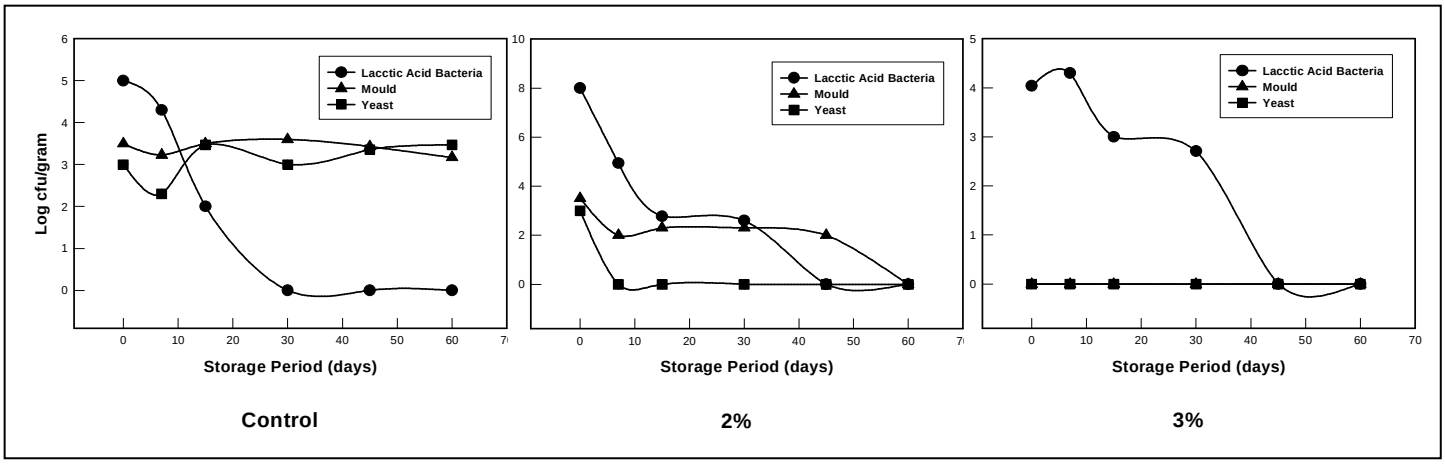


Using Cell Free Supernatant of *P. thoenii*

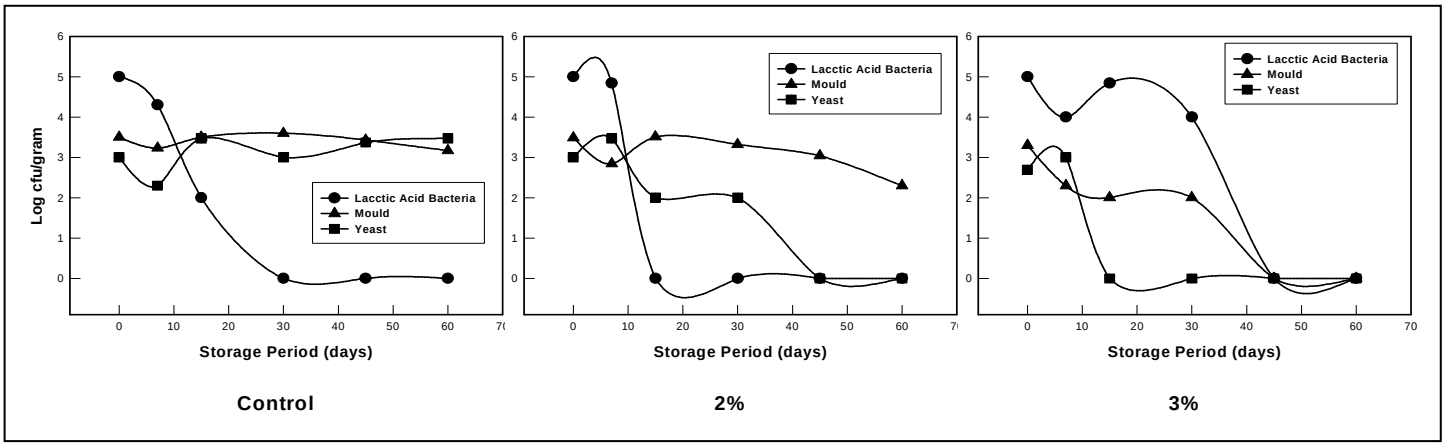


Using Cell Free Supernatant of *P. shermanii*

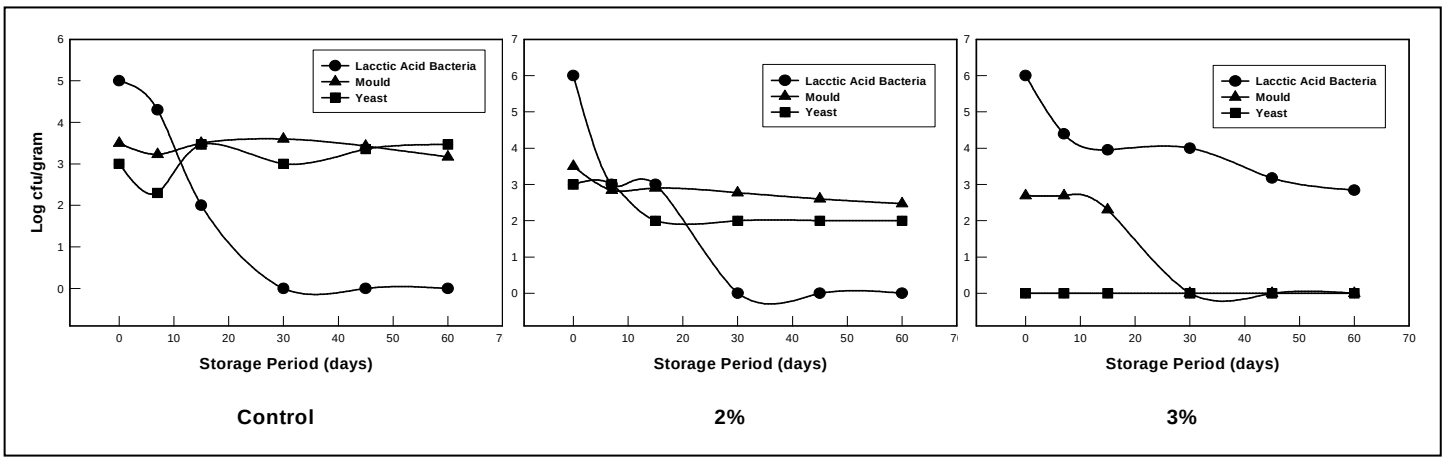
Figure 2: Potential of *L. plantarum*, *P. thoenii* and *P. shermanii* cell free supernatants used in two doses (2% and 3%) to prevent growth of contaminating molds and yeasts and their effect on population of wild lactic acid bacteria in pickled green pepper during storage.



Using Cell Free Supernatant of *L. plantarum*

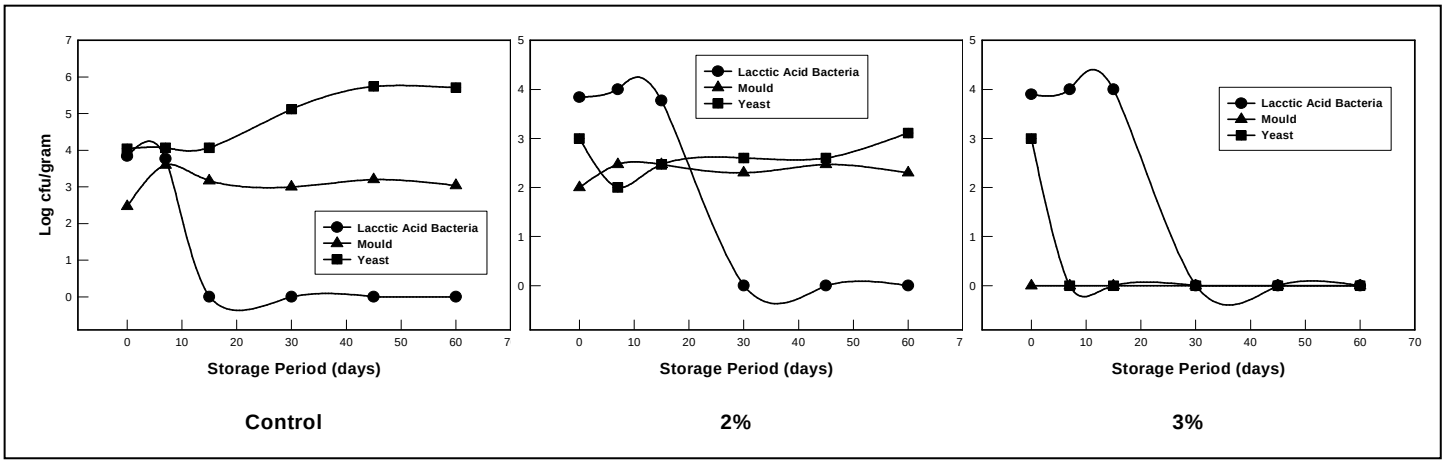


Using Cell Free Supernatant of *P. thoenii*

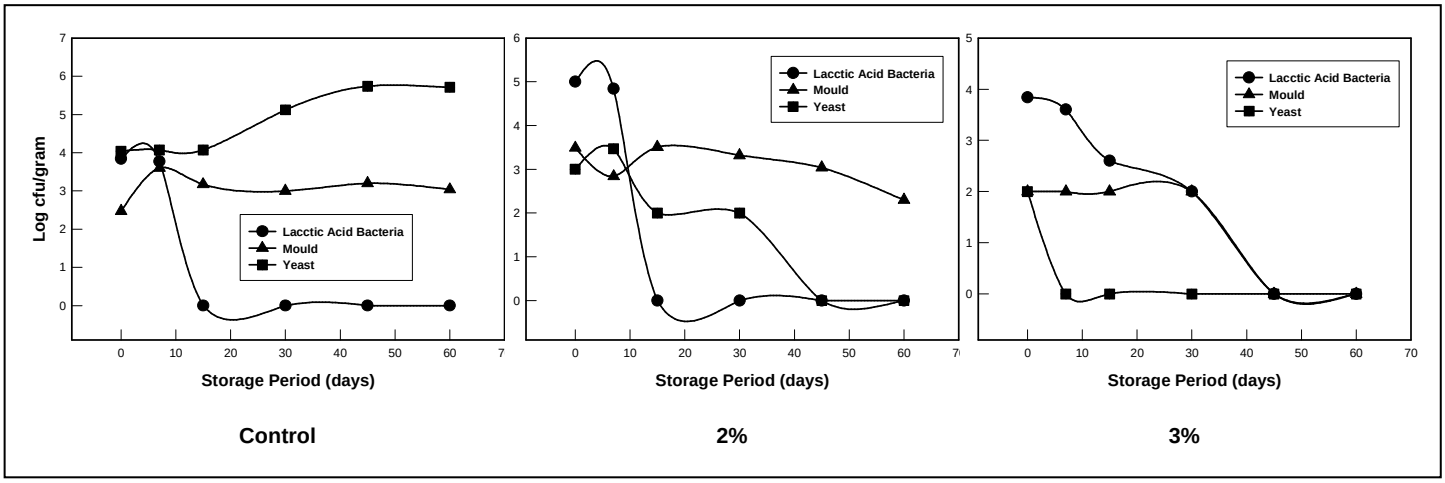


Using Cell Free Supernatant of *P. shermanii*

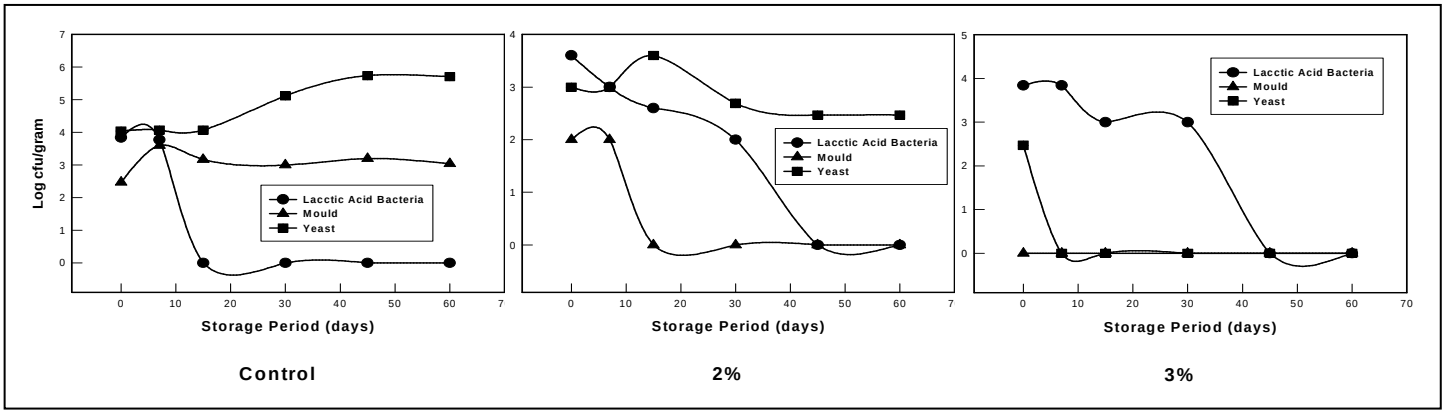
Figure 3: Potential of *L. plantarum, P. thoenii* and *P. shermanii* cell free supernatants used in two doses (2% and 3%) to prevent growth of contaminating molds and yeasts and their effect on population of wild lactic acid bacteria in pickled lemon during storage.



Using Cell Free Supernatant of *L. plantarum*



Using Cell Free Supernatant of *P. thoenii*



Using Cell Free Supernatant of *P. shermanii*

Figure 4: Potential of *L. plantarum*, *P. thoenii* and *P. shermanii* cell free supernatants used in two doses (2% and 3%) to prevent growth of contaminating molds and yeasts and their effect on population of wild lactic acid bacteria in pickled cucumber during storage.

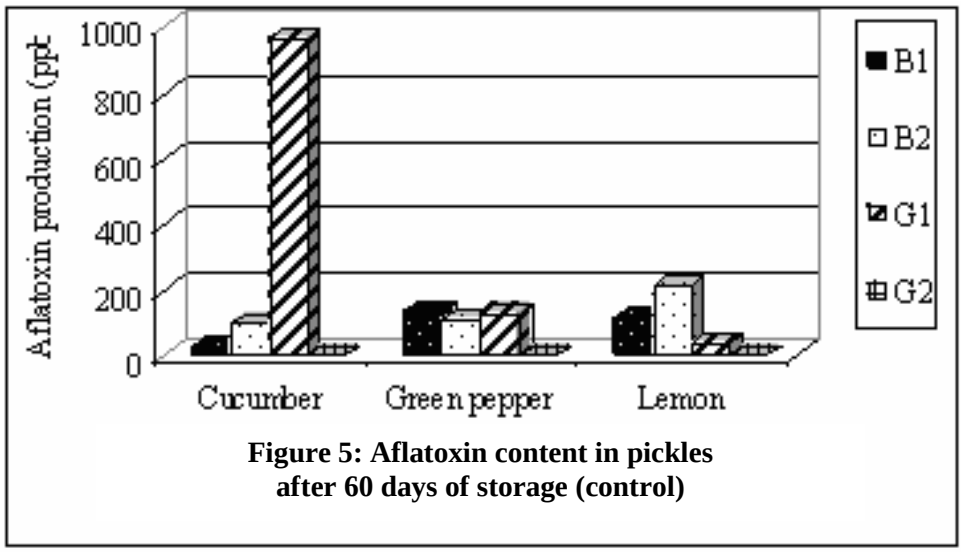


Figure 5: Aflatoxin content in pickles after 60 days of storage (control)

Conclusion

From the above results it could be concluded that LAB and PAB have the ability to produce natural antifungal substances. These metabolites (cell free supernatant) could inhibit mold and yeast growth as will as aflatoxin production. This means that these metabolites could be utilized as anatural preservatives in some foods such as pickles. In addition the inhibition of *A. parasiticus* during pickling prevented the formation of aflatoxin and consequently improved the quality & safety of these products.

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