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# ANTIFUNGAL SUBSTANCES FROM SOME LACTIC ACID BACTERIA AND PROPIONIBACTERIA FOR USE AS FOOD PRESERVATIVES

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## ABSTRACT

The antifungal activity of some lactic acid bacteria and *Propionibacterium thoenii* against some yeasts and moulds was tested by using well agar diffusion assay. Also, the nutritional requirements for the synthesis of antifungal substances (AFS) by tested culture were investigated. Out of the tested cultures, the most promising cultures having a broad spectrum of antifungal activity was *Lactobacillus reuteri* followed by *Lactobacillus acidophilus*. A combination between pairs of AFS was found to be the most active against tested yeasts and moulds. Among six media tested, trypticase soy broth was found to be the best medium for production of antifungal substances by *L. reuteri* and *L. acidophilus*. While, Elliker broth and sodium lactate broth supported maximum production of AFS by *Lactobacillus rhamonsus, Pediococcus acidilactici* and *Prop. thoenii*. Optimum antifungal activity was noticed when *L. reuterii* grown at 37°C for 16 h. Organic nutrients such as glucose and yeast extract, as well as NaCl and $CaCl_2$ influnced the production of antifungal substances when added to the growth medium. Each stimulated production at some concentrations.

The efficacy of antifungal substances was determined to control the yeast and mould in Domiati cheese over long storage (21 d) at 7°C. Shelf-life analysis demonstrated that incorporation of antifungal substance produced by *L. reuteri* at a level of 10% could effectively inhibit the growth of *Saccharomyces cerevisiae* and *Penicillum* spp.

**Keywords:** Antifungal activity, lactic acid bacteria, propionibacteria, yeast, mould, Domiati cheese.

## INTRODUCTION

The contamination of dairy products with undesirable yeasts and moulds is a serious and frequently disturbing problem in dairy industry (Roy *et al.*, 1996). Spoilage of cheese caused by yeasts happens, and occurs as visible growth of yeast colonies on the surface of cheese, as unpleasant smell or taste, as changes in colour and texture or as deformation of the packets containing the cheese. While, mould growth on cheese is a problem during ripening and curing as well as during refrigerated storage (Foster *et al.*, 1957). Species of *Penicillium* and *Aspergillus* are common contaminants of cheese. Until recently mould growth on cheese was considered undesirable primarily for aesthetic reasons, because the growth imparted musty off flavors to the cheese. Some of the moulds contaminating cheese are capable of producing highly potent and extremely carcinogenic mycotoxins and hence could poss serious health problems to the consumer (Bullerman and Olivigni, 1974).

Recently, there has been significant commercial interest in using lactic acid bacteria and propionibacteria as natural food preservatives to enhance food safety and stability as the antimicrobial systems possessed by these bacteria offer potential for effective natural preservation methods (Batish *et al.,* 1997 and Suomalainon and Makinen, 1999). The judicious selection of lactic cultures and propionibacteria capable of producing antifungal substances (AFS) can minimize the yeast and mould contamination in fermented dairy products.

Studies on the antifungal properties of lactic acid bacteria and propioni-bacteria are relatively rare. Few studies reported that supernatant containing acidocin from *Lactobacillus acidophilus* caused a reduction in growth of yeast strains belonging to the genera *Kluyveromyces* and *Canadida*. Also, it caused suppersion of the growth of mould strains belonging to the genera *Penicillium*, *Cladosporium* and *Alteranria* (Collins and Hardt, 1980; Amemiya *et al.,* 1986 and Plockova *et al.,* 1997). Besides, El-Ziney and Debevere (1998) reported that reuterin produced by *Lactobacillus reuteri* exhibits an inhibitory activity against a wide range of microorganisms including yeast and fungi. In a different studies, Vandenbergh (1989) and Lehto and Salminen (1997) reported that *Lactobacillus rhamonsus* has been shown to inhibit growth of some yeasts and moulds. Besides, Mehanna (1999) found that *Pediococcus acidilactici* had an inhibitory effect (Pediocin) on growth of yeast. Moreover, Lyon and Glatz (1993) have shown that bacteriocin produced by *Propionibacterium thoenii* strain P127 was active against some yeasts and moulds.

The first aim of this study was to screen a number of different species of *Lactobacillus*, *P. acidilactici* and *Prop. thoenii* P127 for their ability to exhibit a broad spectrum of antifungal activity, and to determine the optimum conditions for the production of antifungal substances by tested culture. The second aim was to evaluate the effectiveness of antifungal substances to control yeast and mould in Domiati cheese at 7°C under laboratory conditions.

## MATERIALS AND METHODS

**Cultures:**

*Lactobacillus acidophilus* was obtained from Chr. Hansen's Lab. Denmark. Strains of *Lactobacillus reuteri* B-14171, *Lactobacillus rhamnosus* B-445, *Pediococcus acidilactici* B-1153 and *Saccharomyces cerevisiae* Y-2223 were provided by Northern Regional Research Laboratory, Illinois, USA (NRRL). *Propionibacterium thoenii* P-127 was supplied by Department of Food Technology, Propionibacteria Culture Collection, Iowa State University. *Kluyveromyces lactis* and the strains of molds used through this study included *Alternaria* sp., *Penicillium digitatum* and *Aspergillus flavus* were obtained from Dairy Microbiology Lab., National Research Centre.

**Screening for Antifungal Activity:**

The following media were used for cultivation of the lactobacilli, Pediococci and Propionibacterium cultures and for production of antifungal substances (AFS):

1. MRS broth (Deman *et al.,* 1960).
2. Elliker's broth (Elliker *et al.,* 1956).
3. Trypticase soy broth (TSB) (Speck, 1976).
4. Permeate soy broth
5. Sodium lactate broth (NLB) (Rehberger and Glatz, 1998).
6. Reconstituted non-fat milk (11%).

**Assay for Antifungal Activity:**

The antifungal activity was tested by the agar well method as described by Batish *et al.* (1989).

Supernatants (after 48 hrs at 37°C) of tested cultures were centrifuged (4000 rpm/min at 4°C for 10 min.). After removing the cell biomasses the supernatant solutions were adjusted to pH 6.0 and pasteurized to 71°C for 10 min. (Plockova *et al.,* 1997 and Mehanna, 1999). 0.2 ml of each yeast or spore mold suspensions was uniformly spread on the potato Dextrose Agar (PDA)(oxoid) surface. The supernatants (0.1 ml) were added into wells. Plates were incubated at 25°C for 3-5 days and then examined for the appearance of clearance zones around each well containing the supernatants. Zones of inhibition were measured in mm using slide calipers. All assays were performed in duplicate, and the results presented are the means of duplicate trails.

**Effect of Incubation Period, Temperature and Supplements on Antifungal Activity:**

The effect of incubation period was studied by inoculating an active culture of *L. reuteri* (1% inoculum) into flasks of suitable medium. Inoculated flasks were incubated at 37°C for periods of 16, 24, 36, 48 and 60 hrs. Individual flasks were kept for each incubation period. At the end of each incubation period, antifungal activity was determined as previously described.

The effect of incubation temperature was studied in the same way as that described for incubation period, except that the individual inoculated flasks were incubated (in duplicate) for 16 h at 30, 37, 40 and 45°C, respectively. At the end of incubation period, antifungal activity was determined as previously described.

In order to study the effect of different additives on the production of AFS by *L. reuteri*, the following supplements were incorporated in the suitable broth medium at the indicated concentrations.

**-Glucose, NaCl and Yeast Extract:** (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3%).

-**$CaCl_2.2\ H_2O$:** (0, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3%).

**Evaluation of Growth Inhibition in Cheese:**

Domiati cheese was manufactured according to Fahmi and Sharara (1950) using 1.5 ml single strength calf rennet solution/kg of salted milk. The resultant cheese was divided into two main portions. The first portion was inoculated with *S. cerevisiae* suspension to give initial count of $10^4$ cfu/g. The second main was inoculated with *P. digitatum* spore suspension for initial count of $10^3$ cfu/g. Then, each portion was divided into four equal portions. They distributed into 8 sterile plastic screw-cap containers and pickled in its own whey. AFS produced from *L. reuteri*, as described previously under optimum conditions, was added to whey to achieve concentrations of 0 (control), 2.5, 5 and 10% in cheese contaminated with *S. cerevisiae* or *P. digitatum*, respectively. Then, the plastic containers capped and stored at 7°C for 21 days. The experiment was carried out in three replicates.

**Shelf-life Analysis:**

Shelf-life analysis was conducted on the samples, at first twice a week and later at weekly intervals. The yeast count was determined on malt extract agar (oxoid) supplemented with tetracycline at a final concentration of 10 mg/liter (Sarais *et al.,* 1996). Mould was counted on potato dextrose agar (oxoid) acietfied to pH 3.5 with sterile lactic acid solution (10%) (APHA, 1992). Plates were incubated at 25°C for 4-5 days. pH values were measured using a digital pH meter model Hanna HT 4817.

## RESULTS AND DISCUSSION

**I. Screening for Antifungal Activity:**

A primary screening was performed using four lactic acid bacteria and one propionibacteria strains to determine if any demonstrated inhibitory activity against a range of yeasts and moulds as indicator strains. Table (1) shows that all tested strains were found to produce inhibitor(s) with variable spectrum activity against indicator cells. Among the tested cultures, *L. reuteri* had the greatest antifungal activity followed by *L. acidophilus*. *L. reuteri* shows a strong antifungal activity against one of the mould cultures, viz. *P. digitatum*. Among the yeast cultures used as indicator strains, the most sensitive toward antifungal substance produced by *L. reuteri* was *S. cerevisiae*. The extent of inhibition differed with the type of yeast or mould used as indicator organisms as has been shown in Table (1). The results obtained are in agreement with those obtained by Axelsson *et al.* (1989) and Chung *et al.* (1989).

All AFS combinations enhanced antifungal activity against all tested yeasts and moulds (Table 2). The results obtained are in agreement with the observations of Schillinger *et al.* (1996) who reported that the antimicrobial efficiency of a bacteriocin may be enhanced or broadened by using it in combination with other bacteriocins. Also, Suomalainen and Makinen (1999) found that a combination of *L. rhamonsus* and *Prop. freudenreichii* spp. *shermanii* inhibited the growth of spoilage yeasts and moulds much more strongly than either of the strains used singly.

**Table 1: Screening of some lactic acid bacteria and propionibacteria for antifungal activity.**

| Organisms | Inhibition Zone Diameter (mm) | | | | |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** |
| *Saccharomyces cerevisiae* | 6 | 11 | 8.5 | 5 | 6 |
| *Kluyveromyces lactis* | 5 | 4 | 5 | 3 | 10 |
| *Alternaria* sp. | 12.5 | 15.5 | 2 | 7 | 8 |
| *Penicillium digitatum* | 14.5 | 16 | 13 | 3 | 12 |
| *Aspergillus flavus* | 3 | 2.5 | 5 | 2 | 4 |

**1. *L. acidophilus*** **2. *L. reuteri***
**3. *L. rhamnosus*** **4. *P. acidilactici***
**5. *Prop. thoenii***
***These data were the average of duplicate trials.**

**Table 2: Effect of combination of pairs of AFS on some yeasts and moulds.**

| Organisms | Inhibition Zone Diameter (mm) | | | | |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** |
| *Saccharomyces cerevisiae* | 12 | 10 | 9 | 14 | 20 |
| *Kluyveromyces lactis* | 9 | 14 | 7 | 11 | 8 |
| *Alternaria* sp. | 20 | 18 | 13 | 12 | 10 |
| *Penicillium digitatum* | 16 | 15 | 20 | 10 | 16 |
| *Aspergillus flavus* | 6 | 7 | 8 | 9 | 7 |

**1. *L. acidophilus + L. reuteri*** **2. *L. acidophilus + L. rhamonsus***
**3. *L. reuteri + L. rhamonsus*** **4. *L. acidophilus + P. acidilactici***
**5. *L. rhamonsus + Prop. thoenii***
***These data were the average of duplicate trials.**

**II. Effect of Growth Medium:**

Results of the effect of different media on production of AFS by lactic acid bacteria and propionibacteria are shown in Table (3). Of the six media, trypticase soy broth allowed maximum production of AFS followed by MRS, Elliker broth and permeate soy broth for *L. acidophilus* and *L. reuteri*. These findings are corroborate those of Batish (1990a&b) and Paik (1996). On the other side, Elliker's broth was found to be the best medium for production of the antifungal substances by *L. rhamonsus* and *P. acidilactici*. Besides, it was found that sodium lactate broth allowed good production of AFS by *Prop. theonii*. Using reconstituted milk for production of AFS resulted in slight inhibition of growth of tested yeast and mould. This indicated that AFS might be produced in reconstituted milk also but the concentration was too low to be detected by the agar well method. This finding was also recorded by Batish *et al.* (1990a&b).

From the foregoing results, it was observed that the growth medium plays a very important role in the production of microbial metabolities under different conditions.

Inasmuch as *L. reuteri* showed the highest antifungal activity, it was selected for further studies. Also, since it produced the greatest amount of AFS in TSB when compared to other media, this medium was selected as the basal medium in the next part.

**Table 3: Effect of different media on production of antifungal substances by some lactic acid bacteria and propionibacteria.**

| Organisms | Media | Inhibition Zone Diameter (mm) | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| *Saccharomyces cerevisiae* | MRS | 7 | 10 | 17 | 6 | ND |
| | Elliker broth | 10 | 10 | 17 | 8 | ND |
| | Trypticase soy broth | 12 | 27 | 13.5 | 3 | ND |
| | Premeate soy broth | 5 | 3 | 3 | 2 | ND |
| | NLB | ND | ND | ND | ND | 5 |
| | Skim milk | 10 | 7 | 3 | 2.4 | ND |
| *Penicillium digitatium* | MRS | 15 | 13 | 15 | 2 | ND |
| | Elliker broth | 2 | 6 | 21 | 5 | ND |
| | Trypticase soy broth | 16 | 35.5 | 10 | 2 | ND |
| | Premeate soy broth | 15 | 2 | 6 | 1.5 | ND |
| | NLB | ND | ND | ND | ND | 13 |
| | Skim milk | 0.5 | 5 | 12 | 1 | ND |

**1.*L. acidiophilus*** **2.*L. reuteri***
**3.*L. rhamnosus*** **4.*P. acidilactici***
**5.*Prop. thoenii***
**ND:Not detected**

***These data were the average of duplicate trials.**

### III. Effect of Incubation Periods and Temperatures:

As seen from Fig. (1) the optimum incubation period and temperature for the maximum production of AFS from *L. reuteri* appeared to be 16h at 37°C which resulted into a large inhibition zone against tested yeast and mould. However, when incubation temperature and period were raised to 45°C and 60h (Fig. 1-a&b), there was an abrupt loss in the antifungal activity indicating unsuitability of higher temperature and incubation period for the production of antifungal substance. The reduction in the AFS after prolonged incubation, as observed in this investigation could be attributed to the conversion of AFS to other metabolities or the enzymatic degradation (Batish *et al.,* 1990b and Gourama and Bullerman, 1995). Results in the current study parallel those reported for antimicrobial substances by other investigators (El-Ziney *et al.,* 1998).

**Fig. 1-a: Effect of incubation periods and 37ºC on antifungal activity of *L. reuteri.***

**Fig. 1-b: Effect of incubation temperatures on antifungal activity of *L. reuteri during 16 h incubation.***

**Fig. 1: Effect of incubation periods and temperatures on antifungal activity of *L. reuteri.***

**IV. Effect of Nutritional Factors:**

**Glucose:**

Maximal synthesis of AFS was noted by *L. reuteri* when glucose was incorporated into the medium at the level of 0.5% (Fig. 2). Results of the present study corroborate findings of El-Ziney *et al.* (1998).

**NaCl:**

Increase in the concentration of salt up to 3% caused a gradual increase in the amount of AFS produced (Fig. 3). These results indicated that sodium chloride at 3% level enhanced antifungal activity. This could be due to a synergistic effect of salt, acids and AFS (Batish *et al.,* 1990a). Similar results were reported by El-Ziney and Debevere (1998).

**Yeast extract:**

The incorporation of yeast extract in the basal medium stimulated the yield of AFS (Fig. 4); maximal yield was recorded at 1.5% yeast extract.

**$Ca^{++}$:** The production of AFS was stimulated at 0.05 to 0.2% $CaCl_2$ when incorporated in broth medium with maximum elaboration at 0.15% (Fig. 5). However, at concentrations exceeding 0.2%, an appreciable decrease in the production of AFS was observed.

**Effect of AFS on the Growth of *S. cerevisiae* and *P. digitatum* in Domiati Cheese:**

Firstly, the results showed that the presence of 2.5 and 5% AFS levels did not cause an appreciable difference in the yeast and mould growth over the control.

The behavior of yeast and mould in Domiati cheese as affected by the addition of 10% AFS from *L. reuteri* is shown in Fig. (6). It is clear that the addition of AFS led to a reduced viability of both tested organisms. The reduction in the *S. cerevisiae* culture was nearly similar to the culture of *P. digitatum*. The populations of *P. digitatum* decreased by 0.65 log cycle after Fhree weeks, whereas *S. cerevisiae* decreased by 0.79 log cycle during the same time. Therefore, strains of *s. cerevisiae* and *P. digitatum* did not differ in their sensitivity to AFS, and a concentration of 10% was shown to be sufficient to stop the growth of tested strains. Since the numbers stayed relatively constant during storage. At the end of storage period, control cheeses were completely deteriorated.

The addition of AFS did not affect the initial pH of Domiati cheese. The pH values decreased from 5.93 to 4.82, 4.67, 5.00 and 5.10 after 21 days for control and AFS treated cheeses, inoculated with yeast and mould respectively (Fig. 6-a&b). The presence of AFS in cheese did not allow the yeast and mould to reduce the pH as much as when the AFS was absent.

In the present study, addition of 10% of AFS to cheese resulted in a number decimal reduction, in yeast and mould numbers, less than those observed by Plockova *et al.* (1997) for *Penicillum* sp. They found that cfu of *Penicillum* sp. decreased by 1.5 log cycle after 36h at room temperature when 5% of acidocin CH5 containing supernatant was added to the liquid medium. The differences between these results are likely to be explained by variations in the experimental conditions, e.g. temperature, contamination

**Fig. 2: Effect of glucose concentration on production of antifungal substance by *L. reuteri.***

**Fig. 3: Effect of sodium chloride concentration on production of antifungal substance by *L. reuteri.***

**Fig. 4: Effect of yeast extract concentration on the production of antifungal substance by *L. reuteri.***

**Fig. 5: Effect of calcium chloride on the production of antifungal substance by *L. reuteri.***

**Fig. 6-a: On the growth of *S. cerevisiae***

**Fig. 6-a: On the growth of *P. digitatum***

**Fig. 6: Effect of AFS from *L. reuteri* on the growth of *S. cerevisiae* and *P. digitatum* in Domiati cheese at 7°C.**

level, type of medium and the type of antifungal substance used. Antimicrobials are less effective inhibitors in the food matrix than in artificial media, as they are susceptible to enzymatic degradation and nonspecific binding to proteins and lipids (Chumchalova *et al.,* 1998). Their effectiveness can also be influenced by the preservation method applied and evaluation of the antimicrobial effect of a bacteriocin requires testing in complex food systems (Shelef and Seiter, 1993).

The results of this study indicate that antifungal substance produced by *L. reuteri* could be used as a biopreservative agent against yeasts and moulds such as *S. cerevisiae* and *P. digitatum* in soft cheeses like Domiati cheese stored under refrigeration. However, novel approaches are needed to increase the effectiveness of antifungal substances as biopreservative agents in foods.

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## المواد ذات التأثير المثبط للفطريات المنتجة ببكتريا حمض اللاكتيك والبروبيونيك لاستخدامها كمواد حافظة للأغذية

**باهر عبد الخالق عفت**
**قسم الصناعات الغذائية والألبان ـ المركز القومى للبحوث ـ الدقى ـ مصر**

تم فى هذا البحث دراسة التأثير المثبط لبعض سلالات بكتريا حمض اللاكتيك والبروبيونيك على بعض الفطريات والخمائر0 كما تم دراسة تأثير العوامل البيئية والغذائية على إنتاج ونشاط هذه المواد المثبطة للفطريات والخمائر 0

وقد أظهرت النتائج أن ميكروب *L. reuteri* كان أكثر الميكروبات المختبرة إنتاجاً للمواد المثبطة للفطريات ويليه ميكروب *L. acidophilus* وعند دمج هذه المواد فى ازواج وجد أن تأثيرها يتزايد ضد الفطريات والخمائر 0 وقد وجد أن بيئة التربتون صويا السائلة كانت أحسن بيئة لإنتاج المواد المثبطة للفطريات بواسطة *L. reuteri* و *L. acidophilus* 0 بينما كان الإنتاج الأمثل لهذه المواد بواسطة *L. rhamonsus* و *P. acidilactici* عند تنميتها على بيئة *Elliker* وأظهر ميكروب *Prop. thoenii* نشاطاً ملحوظاً عند تنيته على بيئة لاكتات الصوديوم السائلة 0 وقد لوحظ أن النشاط الأمثل للمواد المثبطة للفطريات والمنتجة بواسطة ميكروب *L. reuteri* يكون عند تنميته على درجة حرارة 37 °م لمدة 16 ساعة0 وقد وجد أن كل من الجلوكوز ومستخلص الخميرة وكلوريد الصوديوم وكلوريد الكالسيوم كان له تأثير على إنتاج ونشاط المواد المثبطة للفطريات 0

وقد دلت النتائج على أن إضافة المواد المثبطة للفطريات المنتجة بواسطة ميكروب *L. reuteri* بنسبة 10% إلى الجبن الدمياطى أدى إلى تثبيط نمو كل من خميرة *S. cerevisiae* و فطر *Penicillium* وإطالة مدة الحفظ 0