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# EVALUATION OF THE USE OF SOME BIFIDOBACTERIAL STRAINS TO PRODUCE A HEALTHY FERMENTED SOYMILK

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## ABSTRACT

**Four strains of bifidobacteria, *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium longum* and *Bifidobaccterium bifidium* were evaluated for their use as starter culture to produce a healthy fermented soymilk. The tested strains were grown on MRSL broth and MRSL broth with 0.3 % bile salts , *B. angulatum* showed the highest growth rate followed by *B. longum* and *B. bifidium*, with *B. adolescentis* being the lowest. Including bile salt (0.3%) in the MRSL broth, although growth was reduced , but all species exhibited primarily some degree of bile tolerance. *B. angulatum* and *B. longum* were more resistant to bile salts than the other two species. Adhesion of bifdobacteria to columnar epithelial cells of the small intestine of sheep was tested. It appears that *B. longum* had better adhesion than the others. Activity of the bifidobacteria and *Streptoccus thermophilus* and *Lacotobacillus delbrueckii subsp.bulgaricus* during the fermentation and storage of cultured soymilk for 48 hrs. were assessed by following the development of titrable acidity (TA) and change in pH. In general the pH of soymilk decreased and the (TA) increased as fermentation time increased. It is clear that B. adolescentis (BA) was the most active species as TA increased faster than the other bifidobacteria and *B. longum* was the least active species. Acid production and the decrease in pH in soymilk were comparatively less with combinations of different *Bifidobaccterium* spp together ( 1:1) compared with individual**

**species. All mixed cultures of bifidobacteria and lactic acid bacteria produced more acidity and lower pH value than that produced by single or mixed cultures of bifidobacteria. A mixed culture of *Bifidobacetrium angulatum* and yoghurt culture (1:1) (*S. thermophillus* and *Lactobacillus delbrueckii* subsp *bulgaricus*) or *Bifidobaccterium longum* and yoghurt culture were suggested to be used to produce a health fermented soymilk. bifidobacetria.**

## INTRODUCTION

Soymilk has been given considerable attention as an economical nutritive beverage suitable for overcoing protein malnutrition in infants in developing countries. It could be consumed as such or/and as fermented products with lactic acid bacteria (Sugimata and Van Bren, 1971). Studies indicated that consumption of soy protein decreases total serum cholesterol and minimizes risks of several types of cancers (Andersen, *et al.*, 1995). The positive health benedicts associated with soybeans have greatly increased consumer awareness and have created a large market for soy foods (Liu, 2000). Fermentation of soybean products with lactic acid bacteria has been studied extensively to develop more digestible food such as soy bean yoghurt (Nsofor, *et al.*, 1992).

Bifidobacterium species are a major component of the intestinal flora of healthy humans. It is reported that these organisms can exert benefical effect including the reduction of serum cholesterol and activation of the immune system and inhibition of the growth of potential pathogens that may cause infectious disease in the host (Hirota, 1990; Hughes and Dallas, 1991 and Ishibashi and Shimamura, 1993). Therefore, bifidbacteria are often incorporated in fermented dairy product to increase there therapeutic value (Samona, *et al.*, 1996). For the combination of health benefits of soybean components and the advantages of fermentation by bifidobacteria, soy-yoghurt has been prepared with the fermentation of lactic acid bacteria or bifidobacteria in soymilk (Chou and Hou, 2000).

Today more than 90 probiotic (bifidobacteria-containing) products mostly of dairy origin, are produced world-wide (Molder *et*

*al.*, 1990). The aim of this study was to select some strains of bifidobacteria for the fermentation of soymilk.

## MATERIALS AND METHODS

**Preparation of soymilk:**

Soybean Giza 22 (obtained from the Agriculture Administration, Minia Governorate) was soaked for 24 h at 5°C in tap water . After decanting the water, the soaked soybeans were mixed with tap water at the ratio of 1:3 using a blender. The resultant soymilk was filtered through double cheese cloth ( Chou and Hou , 2000) .

**Starter cultures:**

Yoghurt starter consisted of *Loctobacillus delbrueckii subsp bulgaricus* (EMCC 11102) and *Streptococcus thermophilus* (EMCC 11044) were obtained from Cairo Microbiological Resource Center(MIRCEN) Faculty of Agriculture, Ain Shams University.

Freeze-dried culture of *Bifidbacterium adolescentis* (ATCC 2229), *Bifidobacterium angulatum* (ATCC 2238), *Bifidobacterium longum* (ATCC 2259) and *bifidobacterium bifidum* (ATCC 2203) were also obtained from MIRCEN.

**Activity of starter culture in soymilk:**

Soymilk was sterilized at 120°C for 15 min, cooled rapidly to 37°C. Starter culture was added at a percentage of 3 % The development of acidity and pH were followerd. pH was measured using pH meter. (Model SA 720, USA), and titratable acidity was determined according to Ling (1963).

**Growth media:**

Bifidobacteria were grown in Loctobacilli MRS broth (Oxoid, Basingstoke, UK) supplemented with 5% (w/v) lactose. Solid medium was obtained by adding 1.5% Bacto-agar to the supplemented MRSL broth. MRSL was supplemented with 0.05% (w/v) L-cysteine-HCL as a reducing agent ( Dave and Shah,1996).

**Growth Studies :**

The 4 strains of bifidobacteria were evaluated for growth in MRSL broth (MRSL medium). Starter cultures were inoculated at a percentage of 1% into MRSL broth . Growth was carried out at 37°C and was monitored by recording absorbance at 660 nm (Ultrospec II spedrophotometer, LKB, Biachram, UK).

**Bile salts tolerance:**

Cultures were tested for growth in MRSL broth medium with or without added bile salts (Oxgall Sigma Chemical Co., St. Lois, Mo., USA) according to Gilliliand *et al.* (1984). Freshly prepared cultures were inoculated (1%) into MRSL broth or MRSL broth containing 0.3% bile salts, incubated at 37°C , monitored for growth hourly by measuring the absorptiion at 660 nm. Comparisons among cultures were based on the time required for each culture to increase the absorption at 660 nm by 0.3 absorption unit. Growth curves were plotted and times required for turbidity to reach an optical density of 0.3 was determined.

**Bacterial adhesion to intestinal epithelial cells:**

Adhesion of bifidobacteria to columnar epithelial cells of sheep was tested using the procedure of Fuller (1989). Adhesion was examined by light microscapy of gram strained preparations.

## RESULTS AND DISCUISSON

**Growth of some bifidobacterial strains in MRSL broth:**

The growth in MRSL broth of four *Bifidobacterium* strains is shown in Fig. 1. All species showed a similar growth profile. In all species, a first log phase of growth was observed during the first 12 to 24 h postinoculation and a second log phase started 48 h post-inoculation and continued until 84 h postinoculation after which the growth of bacteria declined. These results agree with those of Tochikura *et al.* (1996) and Al-Salah *et al.* (1998) who attributed this pattern of growth to the presence of two different β-galactosidases. Burification of 2-β-galactosidases from *B. longum* 401 was reported by Tochikura *et al.* (1996). However, *B. angulatum* showed the highest growth rate, followed by *B. longum* and *B. bifidium* with *B. adolescentis* being the lowest. Devries and Stouhamer (1969)

attributed the differences in growth rate among species of bifidobacteria to different levels of tolerance to aerobic condition.

**Bile tolerance of the bifidobacterial strains:**

Including bile salt (0.3 °/o) in the MRSL broth apparently had no effect on the growth profile of all species. Although growth was reduced due to the presence of bile but all species exhibited primarily some degree of bile tolerance (Fig. 2).

*B. angulatum* and *B. longum* were more resistant to bile salts than the other two species. They reached the absorbance of 0.3 nm after 15 and 28 hrs, whereas, it took 40 and 60 hrs for *B. bifidium* and *B. adolescentis* to reach the same absorbance (Fig. 2). Tanaka et al (1999) found that bile sensitivity was different between species. They noted that *B. longum* was more tolerant to bile salts. Also, Joang and Shah (2005) reported that *B. longum* was the best tolerance to bile salts followed by *B. bifidium* and *B. angulatum*. On the other hand, Ibrahim and Bezkorovaing (1993) reported that *B. inifantis* had the best survival rates followed by *B. bifidium*, *B. breve* and *B. longum*. The present results showed that *B. angulatum* was more resistant to bile salt followed by *B. longum* and *B. bifidum* and *B. adolescentis* being the least.

Tahri *et al.* (1995), reported that gram positive bacteria are capable of hydrolyzing the amide bond of conjugated bile salts, liberating free bile salts with lower detergent properties.

**Adhesion to intestinal epithelial cells:**

A major consideration in the choice of *Bifidobacterium* to be used as dietary adjuncts must be the strain that cannot only survive stomach acidity but also establish within the digestive tract. Therefore, the adhesion of bifidobacteria to columnar epithelial cells of the small intestine of sheep was tested. Fig. 3 shows the appearance of the sheep epithelial cells after the removal of the adherent bacteria.

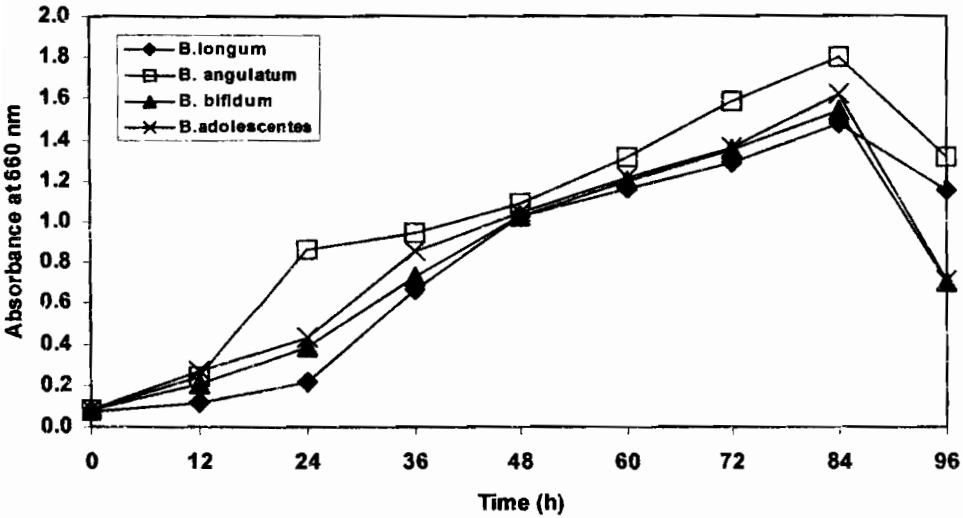


**Fig. 1: Growth curve of different species of bifidobacteria in MRSL.**

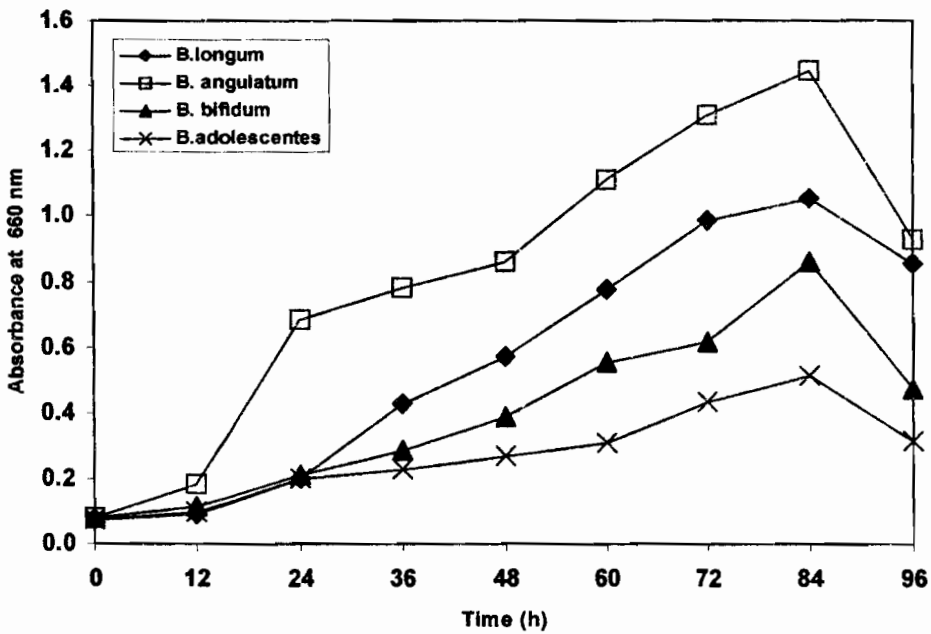


**Fig. 2: Growth of bifidobacteria in MRSL with ( 0.3 %) bile salts.**

Mayra Makinen *et al.* (1983) demonstrated that adhesive bacteria showed a concentration of organisms on the epithelial cells. The adherence of four species of bifidobacteria to sheep epithelial cells is shown in Fig. 4. It appears that *B. longum* (a) had better adhesion followed by *B. angulatum* (b) and *B. bifidium* (c) with *B. adolescentis* (d) being the least. Gilliland *et al.* (1985) reported some differences in the characteristics of organisms isolated from different hosts. The difference between bifidobacteria to human colonic epithelial cells which appeared to be specific, reversible, and depend on the length of contact time and cell concentration (Fischer *et al.*, 1986). The higher the level of the fatty acids fractions of the lipoteichoic acids, the better the adhesion .

**Fig. 3: Sheep cells without bacteria (control).**

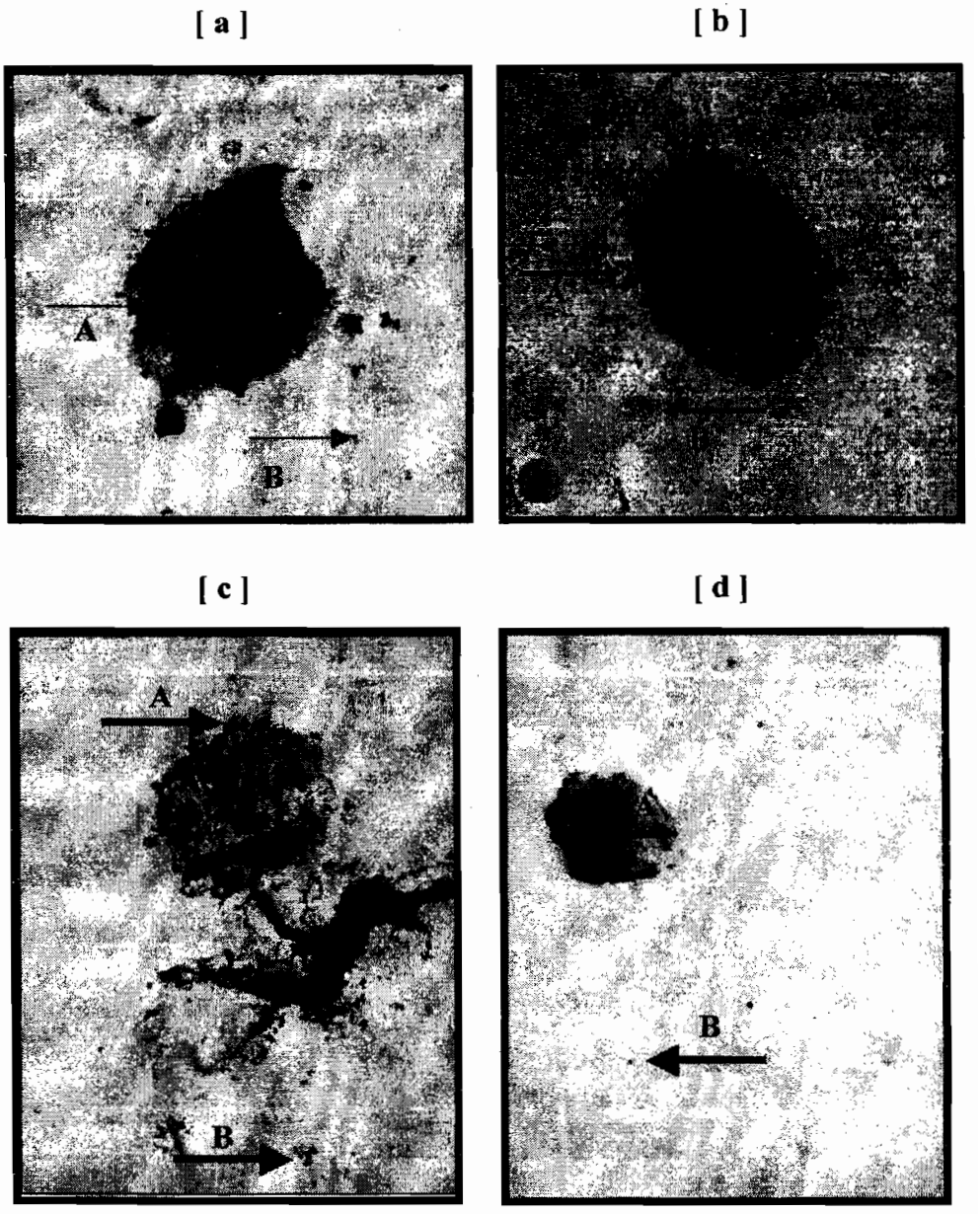


**Fig. 4: Adherence of bifidobacteria to sheep cells. (a) *B. bifidum;* (b) *B. angulatum;* (c) *B. bifidum* and (d) *B. adolescents.***
(A) Adhesive of bifidobacteria. (B) Non adhesive of bifidobacteria.

## Evaluation of some bifidobacterial strains

**Activity in soymilk:**

The activity of *Bifidobacterium* in soymilk was assessed by following the development of titratble acidity and changes in pH. Bifidobacterium culture was added at a percentage of 3 %.

**a) Individual species:**

Results in Table 1 show that pH values and titratable acidity (TA) were quite varied among tested *Bifidobacterium* species, possibly due to the difference in the growth of bifidobacteria in soymilk. As *Bifidobacterium* ssp differ in the nutritional requirements, and the sensitvity to oxygen (Desjardins *et al.*, 1990 and Shimamnra *et al.*, 1992).

In general, the pH of soymilk decreased and the TA increased as the fermentation time increased. It is clear that *B. adolescentis* (BA) was the most active species, as the pH was reduced and TA increased faster than the other three species. This was followed by *B. bifidum* (Bb) then *B. angulatum* (BN) and *B. longum* (BL), being the least active species.

The decrease in the pH and the increase in TA values may be due to capability of bifidobacteria to hydrolyze fructo-oligosaccharide (Mckellar and Molder, 1989). Also soymilk contains sucrose, raffinose and melibiose which bifidobacteria can hydrolyzes an α-galactoside bound in such sugar and produce lactic acid (Roy *et al.*, 1991).

**b) Bifidobacterium mixtures:**

Because of the lack of detailed information in the literature about the behavior of combinations of different *Bifidobacterium* spp together in soymilk. This experiment was carried out to assess the possibility of using a mixed culture of two strains of bifidobacteria in soymilk for acid development. When *B. adolescentis* was used in combination with *B. bifidum* the amount of lactic acid produced and the drop in pH were relatively higher (Table 1). Also, mixture of *B. longum* and *B. bifidium* (BL+Bb) gave comparatively higher acidity and lower pH compared with *B. adolescentis* + *B. angulatum* (BA+BN) and *B. adolescentis* + *B. longum* (BA+ BL) and was more or less closer to *B. adolescentis* + *B. bifidium* (BA+Bb). The amount of acidity and the decrease in pH were the least with *B. angulatum* + *B. longum* (BN+ BL) mixture.

From Table 1, it appears that acid production and the decrease in pH were comparatively less with mixed species compared with individual species. This could be attributed to the competed effects between bifidobacteria when added together, which limit the acidity of each other. The prohibited effect of one species to another could be another factor. Also, Hallingh and Viljoen (2001) observed that when mixed cultures of bifidobacteria were used, the amount of lactic acid produced was lower than using single culture, the inhibition was presumed to be due to antagonism effects among starter bacteria.

**Table 1: pH and titratable acidity (TA) of soymilk fermented with *Bifidobacterium ssp.***

| Organisms | Fermentation time (hours) | | | |
|---|---|---|---|---|
| | 24 | | 48 | |
| | pH | Acidity % | pH | Acidity % |
| Soymilk (control) | 6.67 | 0.13 | 6.67 | 0.13 |
| Soymilk with | | | | |
| BA | 4.52 | 0.58 | 4.28 | 0.69 |
| Bb | 4.54 | 0.57 | 4.31 | 0.67 |
| BN | 4.70 | 0.49 | 4.57 | 0.55 |
| BL | 4.76 | 0.47 | 4.61 | 0.53 |
| BA + Bb | 4.60 | 0.53 | 4.41 | 0.61 |
| BL + Bb | 4.63 | 0.50 | 4.55 | 0.56 |
| BN + Bb | 4.70 | 0.51 | 4.53 | 0.58 |
| BA + BN | 4.76 | 0.46 | 4.57 | 0.55 |
| BA + BL | 4.78 | 0.46 | 4.55 | 0.56 |
| BN + BL | 4.82 | 0.42 | 4.63 | 0.50 |

**BA:** *Bifidobacterium adolescentis.*
**BN:** *Bifidobacterium angulatum .*
**BL:** *Bifidobacterium longum*
**Bb:** *Bifidobacterium bifidum.*

It could be concluded from the practical point of view of the obtained results that, it is preferable to use individual species of bifidobacteria in the dairy industry than mixed ones.

**c) Bifidobacteria with lactic acid bacteria:**

There are many problems associated with the manufacture of fermented products using *Bifidobacterium* spp. One of the most important problems is the slow acid production, which results in a prolonged fermentation time. Samona *et al.* (1996) concluded that, bifidobacteria were rarely employed alone in the production of probiotic yoghurt.

The dairy industry faces this problem by using combined cultures of bifidobacteria and other lactic acid bacteria. The advantage of using a mixed culture containing bifidobacteria and yoghurt bacteria is not only due to the reduction of the fermentation time but also the avoidance of other effects that fermentation products containing only bifidobacteria may have. Therefore, this experiment was designed to examine the influence of certain yoghurt bacteria on the pH and acidity in soymilk fermented by *bifidobacteria* during fermentation. The changes in pH and TA during the fermentation of soymilk inoculated with *bifidobacteria* and lactic acid bacteria were followed and the results are summarized in Table 2.

All mixed cultures of *bifidobacter*ia and lactic acid bacteria produced more acidity and lower pH values than that produced by pure *bifidobacteria* single or mixed cultures (Table 1 and 2). These agree with results obtained by Samona *et al.* (1996) who found the same results and explained that, in mixed culture the level of acid was a replication of the combination of yoghurt culture and *Bifidobacteria* and this observation suggested that there is adgree of influence between the cultures.

A lower pH and higher acidity values were found in soymilk inoculated with mixed culture of different bifidobacteria and *Streptococcus thermophilus* than in soymilk inoculated with *bifidobacteria* and *Lactobacilus delbrueckii* subs. *bulgaricus*. For instance, when soymilk inoculated with *B. adolescentis* and *S. thermophilus* the pH values ranged from 4.38 to 4.09 also, the acidity increased from 0.63 to 0.79 after 24-h and 48-h incubation respectively. But when soymilk inoculated with *B. adolescentis* and *L. delbrueckii* subs. *bulgaricus*, the pH values decreased from 4.58 to 4.36 and the acidity values increased from 0.56 to 0.63 after 24-h and

48-h incubation respectively. This result may be attributed to inability of *L. delbrueckii* subs. *bulgaricus* to utilize sucrose, the main sugar in soymilk (Chou and Hou, 2000). Also, Murti *et al.* (1992) demonstrated that bifidobacteria have a deleterious effect on *L. delbrueckii* subs *bulgaricus* in soymilk, may be to acetic acid production by bifidobacteria (Samona *et al.* 1996; Scalabrini *et al.*, 1998 and Chou and Hou, 2000).

**Table 2: Changes in pH and titratable acidity of soymilk after fermentation with a mixture of *Bifidobacteria* and lactic acid bacteria incubated at 37°C.**

| Organisms | Fermentation time (hours) | | | |
|---|---|---|---|---|
| | 24 | | 48 | |
| | pH | Acidity % | pH | Acidity % |
| BA + S.Th | 4.38 | 0.63 | 4.09 | 0.79 |
| BA + Lb | 4.58 | 0.56 | 4.36 | 0.63 |
| BA+S.Th+Lb | 4.30 | 0.68 | 4.04 | 0.82 |
| BN + S.Th | 4.58 | 0.55 | 4.43 | 0.60 |
| BN + Lb | 4.72 | 0.49 | 4.58 | 0.56 |
| BN+S.Th+Lb | 4.55 | 0.57 | 4.38 | 0.62 |
| BL + S.Th | 4.60 | 0.52 | 4.43 | 0.60 |
| BL + Lb | 4.81 | 0.41 | 4.58 | 0.55 |
| BL+S.Th+Lb | 4.58 | 0.55 | 4.39 | 0.61 |
| Bb + S.Th | 4.43 | 0.60 | 4.20 | 0.75 |
| Bb + Lb | 4.58 | 0.55 | 4.42 | 0.60 |
| Bb+S.Th+Lb | 4.38 | 0.62 | 4.16 | 0.77 |

**S.Th:** *Streptococcus thermophilus* .
**Lb :** *Lactobacillus delbrueckii subsp. bulgaricus* .
**BA:** *Bifidobacterium adolescents* .
**BN:** *Bifidobacterium angulatum* .
**BL:** *Bifidobacterium longum* .
**Bb:** *Bifidobacterium bifidum.*

When a mixture of *B. adolescentis* and yoghurt bacteria were used, the pH values decreased from 4.30 to 4.04 and the acidity values increased from 0.68 to 0.82 after 24-hrs. and 48-hrs. of incubation respectively. However, when soymilk inoculated with mixture of *B. longum* and yoghurt culture the pH values were decreased from 4.58 to 4.39 and the acidity values were increased from 0.55 to 0.61 after 24-hrs to 48-hrs of incubation, respectively.

A mixed culture of bifidobacteria and lactic acid bacteria (*S. thermophilus* and *L. delbrueckii* subs *bulgaricus*) are suggested to be used to ferment soymilk because the pH values were lower and the acidity values were higher than other previous treatments (Table 2).

A mixed culture of *Bifidobacetrium angulatum* and lactic acid bacteria (1:1) (*S.thermophillus* and *Lactobacillus delbrueckii* subsp *bulgaricus*) or *Bifidobaccterium longum* and lactic acid bacteria (*S. thermophillus* and *Lactobacillus delbrueckii* subsp *bulgaricus*) are suggested to be used to ferment soymilk because the pH and acidity values were suitable for making fermented soymilk. The higher ability of *Bifidobacetrium angulatum* and *Bifidobacetrium longum* to grow and their resistance to bile salts and their ability to adhesion than the other species of bifidobacetria.

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**Evaluation of some bifidobacterial strains**

# تقييم استخدام بعض سلالات جنس البيفيدو بيكتريا لأنتاج لبن صويا متخمر صحي

**صباح تونى عبد الرازق، أحمد شوقى زهران،**
**على أحمد محمد متولي ومها محمود السيد بخيت**
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أجريت الدراسة على أربعة أنواع من جنس البيفيدو شملت ، *Bifidobactrrium angulatum, Bifidobactrerium adolescentis Bifidobacterium longum*و *Bifidobacterium bifidium* حيث تم قياس نموهم فى بيئة بكتريا حامض اللاكتيك المدعمة بسكر اللاكتوز وقد وجد أن سلالة *Bifidobactrrium angulatum* كانت اكثرالأنواع سرعة فى النمو وأقل الأنواع سرعة فى النمو كانت سلالة *Bifidobactrerium adolescentis* .

من قياس النمو فى وجود ٣و٠ % ملح الصفراء ووجد أن معدل النمو قد انخفض لجميع السلالات المختبرة ولكن بدرجات متفاوتة من المقاومة لتلك الأملاح وكانت كل من *Bifidobactrrium angulatum* و *Bifidobacterium longum* أكثر البكتريا مقاومة لاملاح الصفراء .

وقد لوحظ أن قدرة خلايا البيفيدو على الالتصاق على الخلايا الطلائية للأمعاء الدقيقة للأغنام وقد وضحت من التجربة أن بكتريا *Bifidobacterium longum* كانت أكثر السلالات قدرة على الالتصاق .

كفاءة نشاط السلالات فى لبن فول الصويا عندما تنمى كسلالة منفردة أو عند خلط سلالتين معا مع بعضهما ، كذلك عند خلط كل سلالة من سلالات البيفيدو على حدة مع بكتريا حامض اللاكتيك *Streptococcus Thermophilus* أو *Lactobacillus delbrueckii subsp. bulgaricus* أو عند خلط كل سلالة من سلالات البيفيدو مع خليط *Lactobacillus delbrueckii subsp. + Streptococcus Thermophilus)*

*bulgaricus*) فى لبن فول الصويا وذلك عن طريق تتبع سير الحموضة وال pH خلال التحضين لفترة زمنية مقدارها ٤٨ ساعة وذلك عن طريق اضافة هذا البادىء بنسبة ٣% وقد وجد أن :

- عند نمو بكتريا البيفيدو منفردة فى لبن فول الصوياكانت سلالةا *Bifidobactrrium angulatum* أكثر السلالات انتاجا للحموضة وانخفاضا فى الpH بينما وجد ان خلط سلالتين من بكتريا البيفيدو مع بعضهما قد أدى الى إنخفاض فى معدل تكوين الحموضة وإرتفاع فى ال pH مقارنة بالسلالة المنفردة مما يفضل معه إستخدام السلالة منفردة من بكتريا البيفيدو .
- لوحظ عند خلط سلالة واحدة من بكتيرا البيفيدو مع بكتريا حامض اللاكتيك Lactobacillus delbrueckii subsp. أو treptococcus thermophilus bulgaricus أن إنتاج الحموضة قد إرتفع وحدث ارتفاع أكبر فى رقم الحموضة للبن فول الصويا مقارنة بالسلالة المنفردة للبيفيدو .

وتوصى الدراسة بأن أفضل السلالات لبكتيريا البيفيدو والتى يمكن إستخدامها مع بكتيريا حامض اللاكتيك *Lactobacillus + Streptococcus thermophilus* *delbrueckii subsp. bulgaricus* للنمو فى لبن فول الصويا هى : *Bifidobacterium longum* أو *Bifidobactrrium angulatum* حيث أنها أكثر السلالات نمواً ومقاومة لأملاح الصفراء وأكثر إرتباط بجدر الأمعاء الدقيقة ، كذلك إنتاجها للحموضة مع سلالات بكتيريا حامض اللاكتيك كان بالدرجة الملائمة التى يمكن معها إستخدامها فى إنتاج لبن فول صويا متخمر صحى.