Bull. Fac. Agric., Cairo Univ.,51 (2000): 473- 488.

GROWTH, BILE TOLERANCE AND ENZYMATIC PROFILES OF VARIOUS SPECIES OF BIFIDOBACTERIA

(Received: 5.2.2000)

By
A. A. Al-Saleh

Department of Food Science and Nutrition, College of Agriculture, King Saud University, Riyadh, Saudi Arabia

ABSTRACT

Six species of bifidobacteria were evaluated for their growth, acid production and viability in supplemented MRS and whole cow milk. Growth rate and acid production of *Bifidobacteria bifidium* and *B. longum* were higher in both media, compared to the other species. The enzymatic profiles of bifidobacteria were determined using the API ZYM test. All species possessed leucine and cysteine arylamidase, ∝- and β-galactosidase and ∝-glucosidase activity. β-glucuronidase was found only in *B. breve*. *Bifidobacteria longum* and *B. bifidium* were more resistant to bile comparing to the other four species. All species were found to be able to adhere to goat epithelial cells.

Key words: *Bifidobacteria , enzymatic patterns , adhesion to epithelial cells .*

1. INTRODUCTION

Bifidobacteria are the predominant gut flora in breast-fed infants and normal components of healthy human intestinal bacteria (Bezkorovainy and Miller-Catchpole, 1989; Rasic and Kurmann, 1983). They were first isolated and described by Tissier (1906). It has been reported that the growth of these bacteria provides protection against intestinal infections(Hughes and Hoover, 1991;Hoover, 1993; Kurmann

1988; Rasic and Kurmann, 1983; Gibson and Wang, 1994). Bifidobacteria produce acetic acid and lactic acid which tend to lower the intestinal pH and thus inhibit the proliferation of pathogenic bacteria (Jiang *et al.*, 1996). Antibacterial properties were also reported by Meghrous *et al.*, (1990) who found that some *Bifidobacterium* spp produced bacteriocins.

The dairy food industry is introducing bifidobacteria to different products such as flavoured milk and cottage cheese. However, fermented dairy products are the most useful media for administering bifidobacteria (Collins and Hall, 1984; Poch and Bezkorovainy, 1988). All the studied bifidobacteria species by Desjardins *et al.*,(1990) showed α- and β-galactosidase activities and α-glucosidase activities. They also found that β-glucuronidase was found in only one strain of the different bifidobacteria tested. These authors concluded that strains of bifidobacteria with high glycosidase activities should be preferred over those with lower activities.

Bile tolerance has been identified as one of the important characteristics that enables probiotics to survive and grow in the intestinal tract (Gilliland, 1979). Therefore, it would be interesting to screen for bile tolerance of different species of *Bifidobacterium*. High growth rate and acidification of milk are also desirable characteristics for selection of bifidobacteria (Kim, 1988; Misra and Muila, 1991). The objectives of this work were to study the growth rate, bile tolerance, acid production, ability to adhere to goat epithelial cells and enzyme profiles of six species of bifidobacteria and their potential use in fermented dairy products.

2. MATERIALS AND METHODS

2.1 Cultures

Bifidobacterium angulatum NCFB 2237, *B. adolescentis* NCFB 2204, *B. bifidum*, NCFB 2715, *B. breve* NCFB 2257, *B. infantis* NCFB 2205 and *B.longum* NCFB 2716 were obtained in lyopholized form from the National Collection of Food Bacteria (Aberdeen, UK).

2.2 Growth media and acid production in milk

The freeze-dried cultures were activated in Lactobacilli MRS broth (Oxoid, Basingstoke, England) supplemented with 5 % (w/v)

Lactose and 0.05 % (w/v) L-cysteine-HCl (Win Lab, Gemini House, Middleessex, Hab 7ET, UK) as a reducing agent. Slant agar was obtained by adding 1.5 % Bacto agar to the supplemented MRS broth. Growth was carried out under anaerobic conditions (Gas Pak System, BBL, Cockeysville, MD, USA).

The fermentation of each strain was determined in pasteurized whole cow milk obtained from King Saud University farm.

2.3 Growth studies

The growth of six cultures of bifidobacteria was monitored in MRS broth supplemented with lactose and cysteine-HCl. Each culture was subcultured twice prior to experimental use. Grov th was carried out, and was measured by recording absorbance at 660 nm (Ultrospec II spectrophotometer, LKB, Biochram, England). One milliliter of the sample was diluted with 0.1 M phosphate buffer (pH 7.0). The pH of the sample was recorded (Corning, pH meter 240).

Growth characteristics of six cultures of bifidobacteria in cow milk were evaluated. Each culture (grown in supplemented MRS to the absorbance of 0.65 at 660 nm) was inoculated at 1 % into 100 ml flasks of pasteurized milk and incubated anaerobically at 37°C for 24 h. Samples were drawn from each flask at 0, 4, 8, 12, 16, 20 and 24 h and gas generating envelope was replaced at each interval. Viable counts were carried out by serial dilution with 0.1 % peptone-water and pour plating in triplicate using supplemented MRS agar (Hughes and Hoover, 1995; Abu-Tarboush *et al.*, 1997).

2.4 Enzymatic profiles

The API ZYM, semi-quantitative micromethod was used (Biomerieux Marcy-L'Etoile, France). The manufacturer's instructions were followed using 4 ml of sterile distilled water as diluent. Colonies from the surface agar plates were suspended in saline to a turbidity corresponding to a McFarland No. 5. API ZYM strips were incubated at 37°C for 4 h. Each strain was tested in triplicate. The enzyme activity level in nanomoles (nm) was measured in accordance with the manufacturer's instruction (Desjardins *et al.*, 1990).

2.5 Bile tolerance

The method of Gilliland *et al.*, (1984) was performed. MRSL was prepared with and without 0.3% oxall (Sigma, St. Louis, Mo, USA). Cultures were inoculated (1%) into MRSL broth in the presence and absence of oxall. The time required for each culture to increase the absorbance at 660 nm by 0.3 absorption units was recorded.

2.6 Adhesion to goat intestinal epithelial cells

The method of Fuller (1973) was used. Segments of duodenum, jejunum and ileum were opened, held in phosphate buffered saline (PBS) pH 7.5 at 4^{O}C for 30 min. The segments washed three times with PBS, and epithelial cells were scraped off with the edge of a microscope slide and suspended in PB. Microscopic examination was used to ensure that the removal of adherent bacteria from cell in the suspensions was achieved.

Bifidobacteria were grown in MRSL broth overnight. The cultures were centrifuged at 4000 x g for 30 min. The supernatant fluid was discarded, and cell pellets were resuspended in PB. One ml of bacterial suspension was added to 1 ml of the epithelial cell suspension.

The mixture was rotated at 20 rev/min at 37^{O}C for 30 min, and adhesion was investigated by light microscopy (1000 x) of Gram stained preparations.

3. RESULTS AND DISCUSSION

Preliminary experiments showed that all studied species reached the end of log phase after approximately 10 - 12 h postinoculation. Table (1) shows growth and acid production of the six species of bifidobacteria in MRSL. Apart from *Bifidobacterium angulatum*, all species showed a relation between growth and acid production. *Bifidobacteria bifidum* and *B. longum* showed the highest growth rate and acid production. These results agree with the findings of Desjardins *et al.*, (1990) and disagree with the observation of Samona *et al.*, (1996) who found that there was uncoupled relation between growth and acid production. All studied species grew well in pasteurized milk. (Figs. 1 and 2). Growth patterns were quite variable, most of them reached the stationary phase after approximately 16 h

postinoculation. *Bifidobacteria infantis* reached the stationary phase after approximately 20 h. The growth rate was higher in *B. bifidum* and *B. longum* compared to the other species. There are inconsistency results regarding the growth of bifidobacteria in milk. Desjardins *et al.* (1991) reported that some strains of bifidobacteria grew well in milk. On the other hand, Klaver *et al.,* (1993) found that the same strains could not grow well in unsupplemented milk. Growth factors have been proposed for optimization of bifidobacterial growth, such as amino sugars, bovine casein digest and yeast extract (Rasic and Kurmann, 1983; Poch and Bezkorovainy, 1988).

Table (1): Growth and final pH of bifidobacteria in supplemented MRSL incubated at 37°C for 12 h.*

Species	pH	Absorbance (660 nm)
B. adolescentis	4.45	2.14
B. angulatum	4.60	3.13
B. bifidum	4.28	3.31
B. breve	4.45	2.09
B. infantis	4.40	2.80
B. longum	4.25	3.40

* (Data are the average of three experiments).

Figures (3) and (4) show the change in pH of pasteurized milk due to the growth of bifidobacteria over a period of 24 h. The initial pH of the milk was 6.58. The change in pH was minimum during the first 8 hours of inoculation, afterwards the decrease in pH was observed till the end of incubation time. The decrease in pH was species dependent. *Bifidobacteria bifidum* and *B. longum* produced more acid than the other species. Scardovi (1986) reported that bifidobacteria have a narrow range of optimum pH for growth (6.5 -7) he found no growth at pH < 5 or > 8.0, whereas Clark *et al.,* (1993) found that *B. longum* could grow at pH 1 and reached 10^2 c.f.u / ml after 3 h of inoculation.

The API ZYM test was used to evaluate the enzymatic profiles of the six species of bifidobacteria (Tables 2 and 3). All studied species possessed acid phosphatase, esterase (C_4 & C_8), leucine arylamidase, cysteine arylamidase and chymotrypsin. Activities of the

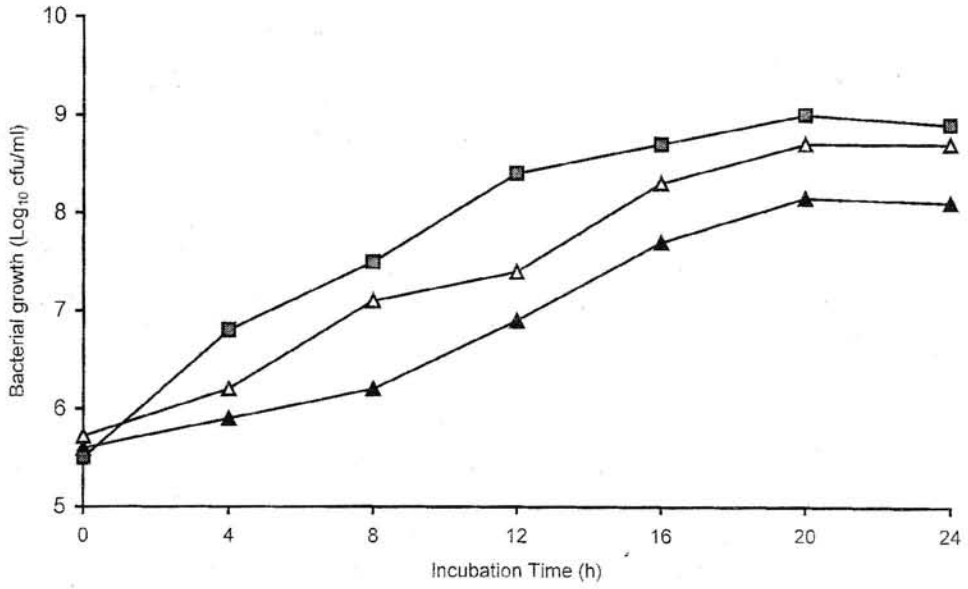


Fig.(1): Growth of (▲ *B.adolescentis*, △ *B. angulatum*, ■ *B. bifidium*) in pasteurized milk incubated at 37°C for 24 h.

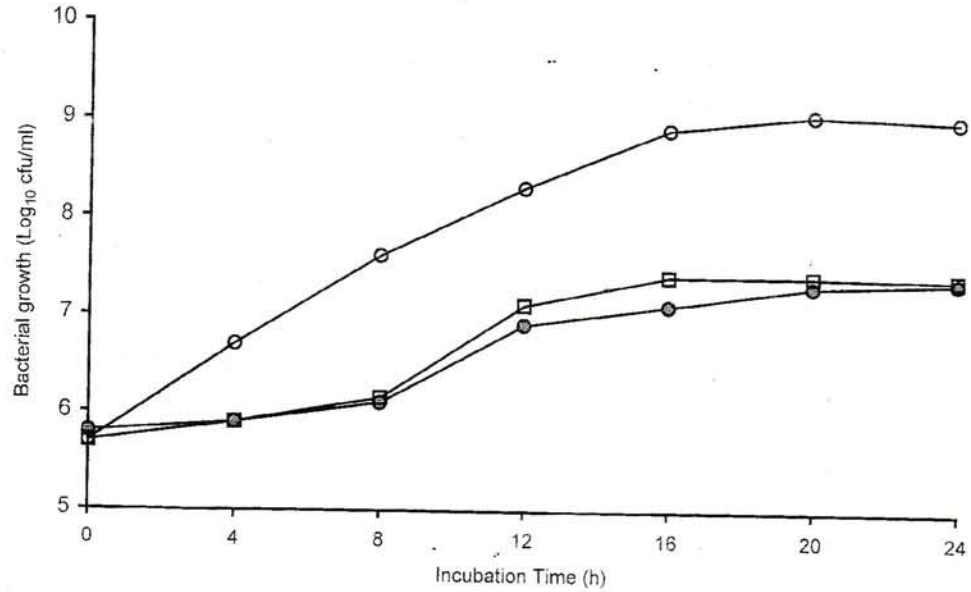


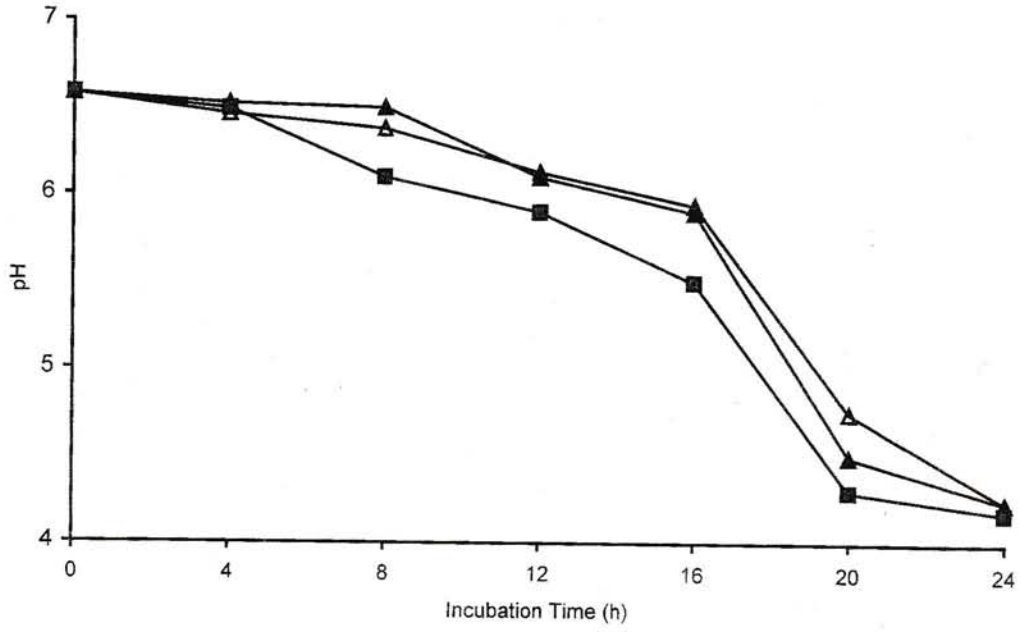
Fig.(2): Growth of (⊟ *B. breve*, • *B. infantis*, ⊖ *B. Longum*) in pasteurized milk incubated at 37°C for 24 h.

enzymes ∝-mannosidase, ∝-fucosidase, lipase (C_{14}) N-acetyl-β-glucosaminidase and trypsin were not detected in any of the six species. The same findings were observed by (Velazquez and Feirtag, 1997). β-glucuronidase was found only in *B. breve* at low activity (5 nM hydrolysed substrate). It was also detected in one strain of *B. breve* ATCC 15699 (Desjardins *et al.*, 1990). This enzyme is one of the fecal enzymes of microbial origin which are thought to convert procarinogens to carinogens (Kurmann, 1988). All studied species had β-galactosidase and ∝-glucosidase activities as high as 40 nM activity level. Bifidobacteria used for therapeutic benefits should possess high β-galactosidase activity (Lin *et al.*, 1991). ∝-galactosidase activity was found to be in the range 30 - 40 nM activity level (Tables 2 and 3). This enzyme is able to utilize nondigestible oligosaccarides, which are not normally utilized by humans. These carbohydrates probably constitute a significant source of carbon for bifidobacteria colonizing the small intestine (Bezkorovainy and Miller-Catchpole, 1989). All bifidobacteria demonstrated ∝-and β-galactosidase and ∝-glucosidase activities (Chevalier *et al.*, 1991).

Table (2):Enzymatic patterns determined with the API ZYM system on (A) *B. adolescentis*, (B) *B. angulatum*, (C) *B. bifidum*[1]

Enzyme assayed	Quantity of hydrolysed substrate (nM)[2]		
	Species		
	(A)	(B)	(C)
Acid phosphatase	≥ 40	≥ 40	≥ 40
Alkaline phosphatase	0	20	10
Esterase (C_4)	10	30	20
Esterase-lipase (C_8)	10	30	10
Leucine arylamidase	≥ 40	≥ 40	≥ 40
Valine arylamidase	0	10	5
Cysteine arylamidase	≥ 40	≥ 40	20
Chymotrypsin	10	20	20
Naphthol-As-BI-phosphohydrolase	20	30	≥ 40
∝-galactosidase	≥ 40	30	≥ 40
β-galactosidase	≥ 40	≥ 40	≥ 40
∝-glucosidase	≥ 40	≥ 40	≥ 40
β-glucosidase	10	10	20

1 (Data are the average of three experiments).
2 Nanomoles.



ig.(3): Changes in pH of (▲ *B.adolescentis*, △ *B. angulatum*, ■ *B. bifidium*) in pasteurized milk incubated at 37°C for 24 h.

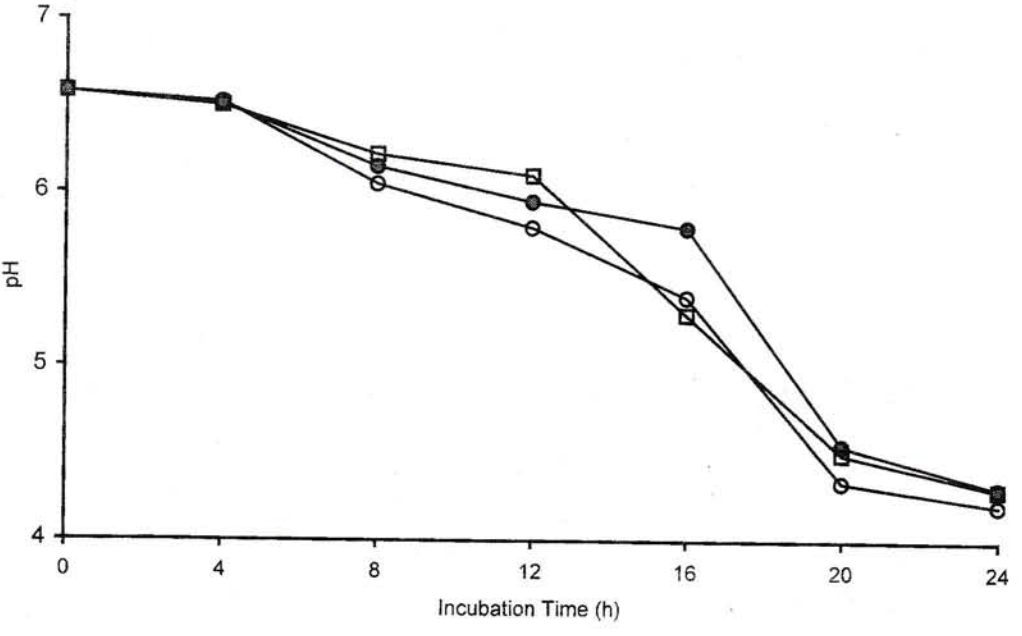


Fig.(4): Changes in pH of (⊟ *B. breve*, • *B. infantis*, ⊖ *B. Longum*) in pasteurized milk incubated at 37°C for 24 h.

Table(3): Enzymatic patterns determined with the API ZYM system on (D) *B. breve*, (E) *B. infantis*, (F) *β. longum*[1]

Enzyme assayed	Quantity of hydrolysed substrate (nM)[2]		
		Species	
	(D)	(E)	(F)
Acid phosphatase	≥ 40	≥ 40	≥ 40
Alkaline phosphatase	10	20	0
Esterase (C_4)	5	20	10
Esterase-lipase (C_8)	10	20	10
Leucine arylamidase	≥ 40	≥ 40	≥ 40
Valine arylamidase	20	0	30
Cysteine arylamidase	30	30	≥ 40
Chymotrypsin	10	10	10
Naphthol-As-BI-phosphohydrolase	≥ 40	20	≥ 40
α-galactosidase	30	≥ 40	≥ 40
β-galactosidase	≥ 40	≥ 40	≥ 40
α-glucosidase	≥ 40	≥ 40	≥ 40
β-glucosidase	10	10	20

1 (Data are the average of three experiments). 2 Nanomoles.

3.1 Bile tolerance

Comparison of the ability of the six species of bifidobacteria to grow in the MRSL broth in the absence and presence of 0.3% oxgall showed considerable variation among species (Table 4) when compared to the control. Oxgall had an inhibitory effect on all species. *β. breve* and *B. adolescentis* were the most sensitive strains towards oxgall. They reached the absorbance of 0.30 after 14 h and 15 h, respectively. *B. longum* and *B. bifidum* were more resistant compared to the other species. Resistance to bile is an important characteristics that enables probiotics to survive and grow in the intestinal tract (Gilliland, 1979). There was a relationship between bile tolerance and β-galactosidase activity. Bile tolerant strains improved the digestion of lactose.

3.2 Bacterial adhesion to intestinal epithelial cells

The adhesion of bifidobacteria to columnar epithelial cells of goat was examined. Figure (5A) shows the appearance of goat

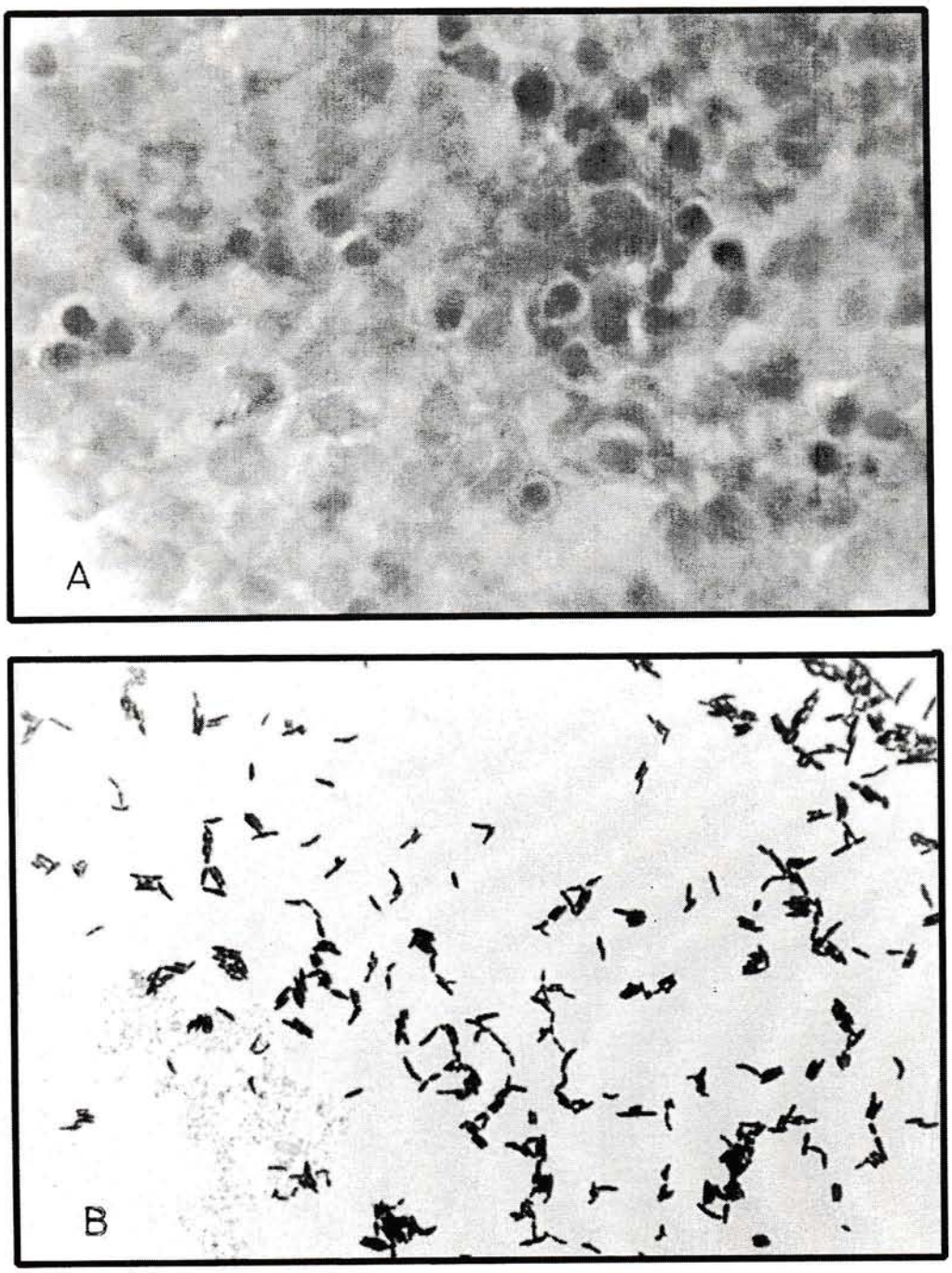


Fig.(5): (A) Goat epithelial cells free from adherent bacteria (B) *Bifidobacterium longum* free from epithelial cells. (1000X).

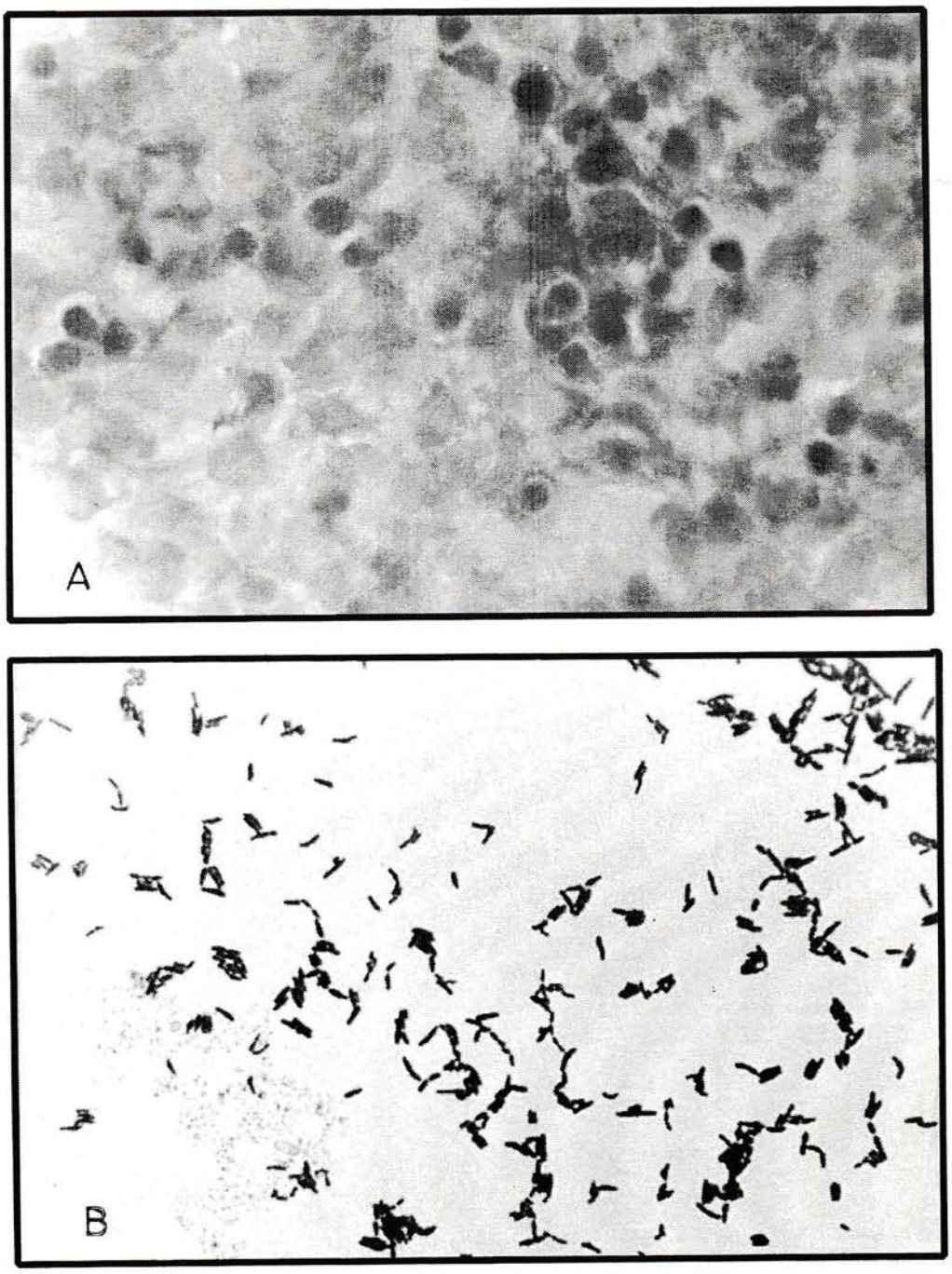


Fig (6):Adhesive of different species of bifidobacteria to goat epithelial cells (a) adhesive bacteria, (b) Non adhesive bacteria (A) *B. bifidum* (B) *B. breve* (1000X).

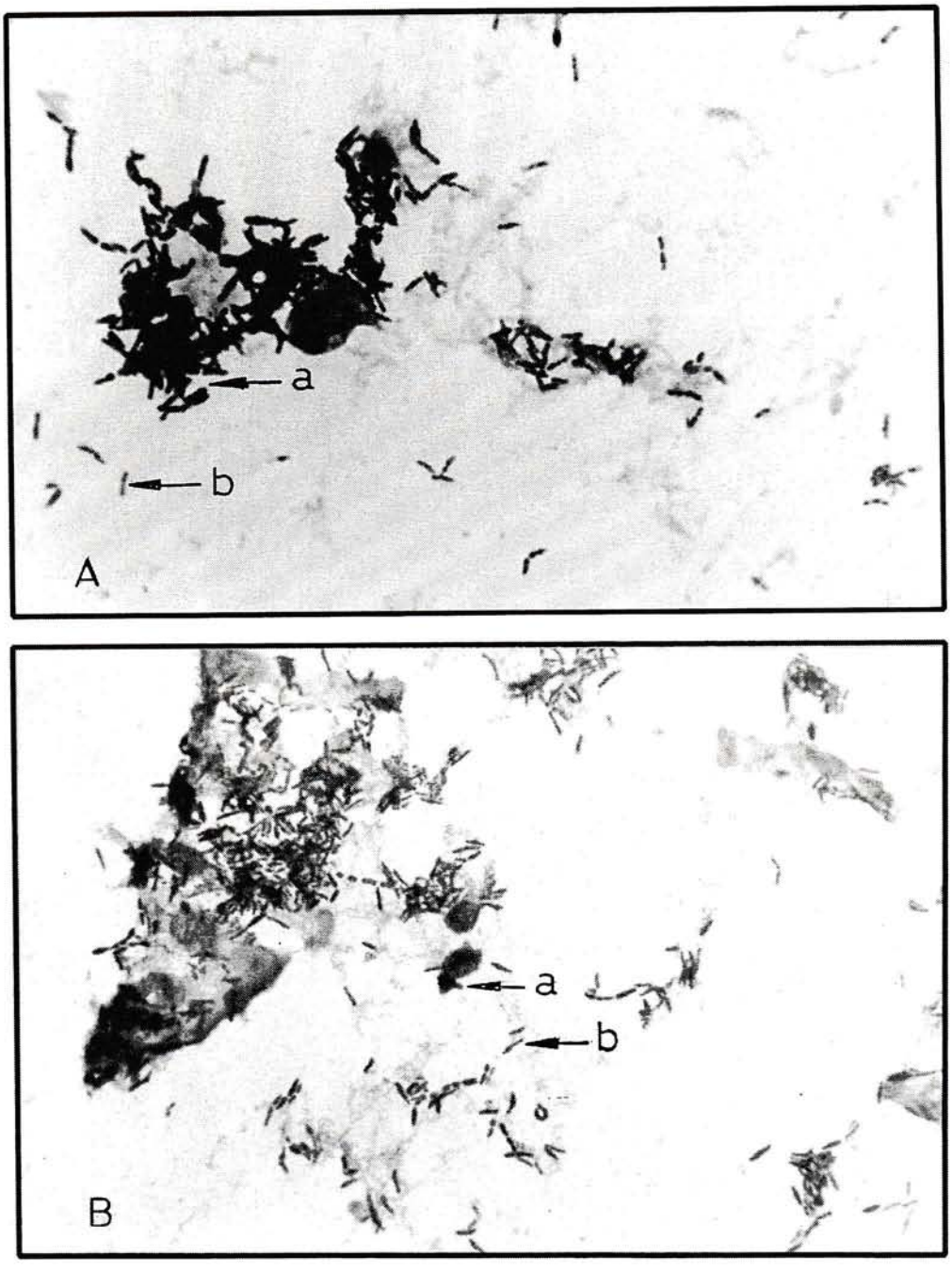


Fig.(7): Adhesive of different species of bifidobacteria to goat epithelial cells (a) adhesive bacteria, (b) Non adhesive bacteria (A) *B. infantis* (B) *B. Longum* (1000X).

epithelial cells after removal of adherent bacteria, wheras Figure (8) shows *B. longum* free from epithelial cells.

Table (4): Comparison of bile resistance of six species of bifidobacteria.*

Species	MRSL broth	Hours to reach A660 nm = 0.3 MRSL broth + 0.3 Oxgall
B. adolescentis	11	15
B. infantis	9	11
B. bifidum	8	9
B. Longum	10	11
B. angulatum	10	13
B. breve	9	14

* (Data are the average of three experiments).

Adhesion to goat epithelial cells has been demonstrated for all species (Figures 6 and 7). It has been reported that the retention of probiotics in the intestinal tract is dependent on absorbtion of bacteria to epithelial surfaces (Fuller,1973). This adherence will allow probiotics to multiply and colonize in the intestinal tract (Conway *et al.*, 1987). Lipoteichoic acids of Gram-positive bacteria have been shown to possess a high binding affinity to mammalian cell membranes (Knox and Wicken, 1973). OpdenCamp *et al.*, (1984) studied the interaction of bifidobacterial lipoteichoic acid with human intestinal epithelial cells. They found that binding of the lipoteichoic acid of *B. bifidum* to human colonic epithelial cells appeared to be specific, reversible, cell concentration and time dependent.

4. REFERENCES

Abu-Tarboush H.M., Al-Dagal M.M.and Al-Royli M.A. (1997). Growth, viability and proteolytic activity of bifidobacteria in whole camel milk. J. Dairy Sci. 81:354-361.

Bezkorovainy A., and Miller-Catchpole E.(1989). Biochemistry and physiology of bifidobacteria. CRC. Press, Bo Ca, Raton, F.L. (USA).

Chevalier P., Roy D. and Savoie L.(1991). X-α-Gal-based, medium for simultaneous enumeration of bifidobacteria and lactic acid bacteria in milk. J. Microbiological. Methods. 13:75-83.

Clark P.A., Cotton L.N. and Martin J.H.(1993). Selection of bifidobacteria for use as dietary adjuncts in cultured dairy food. II. Tolerance to simulated pH of human stomach. Cultured Dairy Products J. 28(4):11–14.

Collins E.B. and Hall B.J. (1984). Growth of bifidobacteria in milk and preparation of *Bifidobacterium infantis* for a Dietary Adjunct. J. Dairy Sci. 67:1376-1380.

Conway P.L, Gorbach S.L. and Goldin B.R. (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci. 70:1-12.

Desjardins M.L., Roy D. and Goulet J. (1990). Growth of bifidobacteria and their enzyme profiles. J. Dairy Sci. 73:299-307.

Desjardins M.L., Roy D. and Goulet J. (1991). β-galactosidase and proteolytic activities of bifidobacteria in milk: a preliminary study. Milchwissenschaft 46:11-13.

Fuller R. (1973). Ecological studies of the *Lactobacillus* flora associated with the crop epithelium of the fowl. J. Applied Bacteriology 36:131-139.

Gibson G.R. and Wang X. (1994). Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J. Applied Bacteriology. 77:412-420.

Gilliland S.E. (1979). Beneficial interrelationships between certain microorganisms and humans: candidate organisms for use as dietary adjuncts. J. Food Protection. 42:164-167.

Gilliland S.E., Staley T.E. and Bush L.J. (1984). Importance of bile tolerance of *Lactobacillus acidophilus* used as dietary adjunct. J. Dairy Sci. 67:3045-3051.

Hoover D.G. (1993). Bifidobacteria: activity and potential benefits. Food Technology. 47:120-124.

Hughes D.B. and Hoover D.G. (1991). Bifidobacteria: their potential for use in American dairy products. Food Technology 45:74-83.

Hughes D.B. and Hoover D.G. (1995). Viability and enzymatic activity of bifidobacteria in milk. J. Dairy Sci. 87:268-276

Jiang T., Mustapha A. and Savaiano D.A. (1996). Improvement of lactose digestion in humans by ingestion of unfermented milk containing *Bifidobacterium longum*. J. Dairy Sci. 79:750-757.

Kim H.S.(1988). Characterization of lactobacilli and bifidobacteria as applied to dietary adjuncts. Cultured Dairy Products J. 23(3):6-9.

Klaver F.A.M., Ginma F. and Weerkamp A.H. (1993). Growth and survival of bifidobacteria in milk. Netherlands Milk and Dairy J. 47:151-164.

Knox K.W. and Wicken A.J. (1973). Immunological properties of teichoic acids. Bacteriological Review. 37:215-257.

Kurmann J.A. (1988). Starter for fermented milks section 5. Starters with selected intestinal bacteria. International Dairy Federation Bulletin. 227:41-55.

Lin M.Y., Savaiano D.A. and Harlander S.K.(1991). Influence of nonfermented dairy products containing bacterial starter cultures on lactose maldigestion in humans. J. Dairy Sci. 74:87-95.

Meghrous J., Eulgo P., Junells A.M., Ballangue J. and Petitemange H. (1990). Screening of *bifidobacterium strains* for bacteriocin production. Biotechnology Letters 12:575-580.

Misra A.K. and Muila R.K. (1991). The selection of bifidobacteria for the manufacture of fermented milks. Australian J. Dairy Technology 46(1):24-26.

OpdenCamp H.J.M., Oosterhof A.and VeerKamp J.H. (1984). Interaction of bifidobacterial lipoteichoic acid with human intestinal epithelial cells. Infection and Immunity 47:332-334.

Poch M. and Bezkorovainy A. (1988). Growth enhancing supplements for various species of the genus *Bifidobacterium*. J. Dairy Sci. 71:3214-3221.

Rasic J.L. and Kurmann J.A.(1983). Bifidobacteria and their role. Birkhauser- Verlag-Basel, Basel, Switzerland.

Samona A., Robinson R.K. and Marakis S. (1996). Acid production by bifidobacteria and yoghurt bacteria during fermentation and storage of milk. Food Microbiology 13:275-280.

Scardovi V.C. (1986). In Bergey's Manual of Systematic Bacteriology, Vol. 2, Pp. 1418-1434 (Eds. Sneath,P.H.A., N.S. Mair, M.E. Sharpe and J.G. Holt). Williams and Wilkins, Baltimore, M.D.

Tissier H. (1906). Traitement des infections intestinales par la methods de transformations de la flora bacterinne de l'intestin. C.R. Soc. Bol. 60:359-362.

Velazquez M. and Feirtag J.M.(1997). Isolation and partial characterization of commercial strains of bifidobateria. J. Food Protection 60(5):537-543.

النمو وتحمل أملاح الصفراء والنشاط الإنزيمي لأنواع مختلفة من البفيدوبكتريا

عبدالرحمن عبدالله الصالح

قسم علوم الأغذية والتغذية ، كلية الزراعة ، جامعة الملك سعود
المملكة العربية السعودية

ملخص

تم تقييم النمو وإنتاج الحمض والنشاط الحيوي لست أنواع من البفيدوبكتريا وذلك في بيئة الـ MRS أو الحليب البقرى. كان النمو وإنتاج الحمض للنوعين *B.longum, Bifidobacteria bifidum* ، أعلى من الأنواع الأربع الأخرى. تم تقدير النظام الإنزيمي لبكتريا البفيدو باستخدام طريقة API ZYM ، أظهرت كل السلالات نشاطاً لكل من الإنزيمات التالية ليوسين وسستين اريل أميديز ، وألفا وبيتا جالاكتوسيديز ، ألفا جلوكوسيديز ، أما إنزيم البيتا جلوكورونيديز فقد وجد فقط في البكتريا *B.breve*. البكتريا *B.longum* و *B.bifidum* أظهرتا تحملا لأملاح الصفراء أعلى من الأنواع الأخرى ، كما أظهرت النتائج عن طريق الفحص الميكروسكوبي أن الأنواع الستة كانت لها قدرة على الالتصاق بالخلايا الطلائية لجدر الأمعاء في الماعز.

المجلة العلمية لكلية الزراعة – جامعة القاهرة – المجلد (51) العدد الرابع (أكتوبر 2000):473-488.