

Acid Tolerance and Growth of Four Strains of Bifidobacteria in Different Types of Milk

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ABSTRACT

Four strains of bifidobacteria (*Bifidobacterium angulatum* ATCC 2238, *Bifidobacterium animalis subsp lactis* Bb12 , *Bifidobacterium bifidum* ATCC 2203, and *Bifidobacterium bifidum* LMG 10645) were studied for acid tolerance and growth in different types of mammalian milks . All studied strains grew at a wide range of pH , ranging from pH 2.0 - pH 6.5 . However the growth was quite low at pH 2.0 , it was in the range between (0.07 - 0.12 optical density at 660 nm) and slightly higher when the pH of the medium was raised to pH 3.0 , at this pH the growth was between (0.08 - 0.18) optical density at 660 nm . On the other hand , at pH 4.0 the optical density was ranging between (0.90 - 2.10 at 660 nm) . The higher growth rate was achieved at pH 6.5 , it was between (3.55- 4.35) optical density at 660 nm. The studied strains were grown in human, cow, goat, buffalo, sheep and camel milks. All strains were able to grow at the six types of milk , the growth was in the range between 1.5×10^6 cfu /ml (sheep milk) - 2.1×10^7 cfu /ml (cow milk) a slight decrease was noticed when *Bifidobacterium angulatum* 2238 was grown in sheep milk . *Bifidobacterium bifidum* 2203 reached the count of 10×10^9 cfu /ml in goat milk and the lowest growth was noticed in human milk . The slowest growth of *Bifidobacterium animalis subsp lactis* Bb12 was 7.5×10^5 cfu /ml , it was found when it was grown in camel milk and the best growth was noticed in goat and sheep milks . *Bifidobacterium bifidum* LMG 10645 grew well in all types of milk except human milk , the growth rate was almost around $10^6 - 10^7$ cfu /ml , whereas a slight decrease was noticed when they grew in human milk 5.9×10^6 cfu /ml.

Keywords : Bifidobacteria , acid tolerance, cow, goat , buffalo and camel milks.

INTRODUCTION

Bifidobacteria are the predominant organisms in the large intestine of breast fed infants accounting for about 99% of the cultivatable flora . A number of health benefits have been claimed for live probiotic bacteria such as immunity system promoting (Saraf *et al.*, 2010) , reduction of lactose intolerance (Kim and Gilliland, 1983) , inhibition of certain diseases (Ershidat and Mazzahereh, 2009) , reduction of cholesterol level (Ziemer and Gibson,1998) and production of vitamins mainly the B group (Moslemi *et al.*, 2016) .

Mammalian milk promotes the development of favourable intestinal bacteria that can protect the intestinal tract from the proliferation of pathogenic bacteria . Mammalian milk is a heterogeneous complex of biological substances such as saccharides, mainly oligosaccharides, amino acids, essential nutrients, vitamins, and minerals, all of which promoting the development of gastrointestinal microflora in the newborn babies during the first few days of its life. There are multiple variances between human and other kinds of mammalian milk (Ročkova *et al.*, 2013).

Mendoza *et al.*, (2007) reported that more than 70 products from dairy origin containing *Lb. acidophilus* and bifidobacteria, are produced world-wide including sour cream, buttermilk, yogurt, milk powder, and frozen desserts . Recently, the key growth sector has been probiotic drinks . Bifidobacteria are nutritionally fastidious microorganisms that require specific growth factors as only a limited number of these bacteria can grow in minimal culture conditions. The objective of this study was to investigate acid tolerance and the growth of four strains of bifidobacteria in different types of milk .

MATERIALS AND METHODS

Microorganisms

Freeze-dried cultures of Bifidobacteria were obtained from Cairo Mircen (Ain Shams university, Cairo, Egypt) , *Bifidobacterium angulatum* ATCC 2238, *Bifidobacterium bifidum* ATCC 2203, *Bifidobacterium animalis subsp lactis* Bb12 and *Bifidobacterium bifidum* LMG 10645.

Milk samples :

Breast milk samples were collected from healthy volunteers in Minia general hospital and also from Minia university hospitals , (pediatrics department). Fresh cow, buffalo and sheep bulk milk samples were obtained from the herds of animal production , Faculty of Agriculture , Minia university. Goat milk was collected from small preeders in Minia governorate. Camel milk , freezing samples were transferred in ice tank from Halayeb &Shalateen - Aswan- Egypt .

Acid tolerance

Ten ml of MRS broth medium adjusted at pH 2.0 , 3.0 , 4.0 and 6.5 pH were prepared. After sterilization, the test tubes were inoculated with 1% v/v fresh liquid cultures of the probiotic bacteria (in three replicates). The bacterial growth was determined by measuring the optical density (O.D 660nm) at zero, 12,18 24,36,48,60 and 72 hrs of incubation at 37°C (Al-Saleh *et al.*,1998) .

pH measurement

pH was measured according to Ling ,(1963) using a pH meter (Model SA 720, USA).

Growth of bifidobacteria in milk

The various milk types were heated to 80°C for 20 min and kept at 4°C until use (Turroni *et al.* , 2011). Each milk sample was inoculated with 1% v/v from a culture which previously activated for 24 hrs . One ml of each sample was serially diluted with 9 ml of 0.1 % peptone water. Appropriate dilutions were plated on MRS+ cysteine-HCl and incubated anaerobically at 37° C for 24 hrs. The total count was recorded (Standard method of the examination of dairy products ,1978).

Results and Discussion:

1- Growth of bifidobacterial in different pH values:

Growth of the selected four bacterial strains at pH 2.0 , 3.0 , 4.0 and 6.5 was measured after incubation for 72 hrs at the optimum temperature (37°C). Growth of each isolate was expressed in terms of optical density at 660 nm .

Data in Fig (1) indicate that all of the selected strains can grow at the different pH values . *Bifidobacterium animalis subsp lactis* Bb12 was the most tolerant strain to pH 4 (2.10) followed by *Bifidobacterium bifidum* 2203 (1.80),

and *Bifidobacterium angulatum* 2238 (1.50) , however *Bifidobacterium bifidum* LMG 10645 (0.91) was the lowest in terms of acid tolerance . These result are an accordance with results of Truelstrup *et al.*,(2002) who reported that *B. lactis Bb12* had high resistance to acid at pH 3.0 .

At pH 3.0 *Bifidobacterium bifidum* LMG 10645 was the most tolerant one (0.18) optical density followed by *Bifidobacterium bifidum* 2203 (0.16) , and *Bifidobacterium animalis subsp lactis Bb12* (0.13) ,and *Bifidobacterium angulatum* 2238 (0.08) . The growth was decreased in all strains at pH 2.0 and pH 3.0 . These observations were in good agreement with what was found by Jia *et al.*, (2009) who studied the growth of four *bifidobacterium* strains at pH between 3.0 - 4.5 , they found that *Bifidobacterium bifidum* was the most tolerant strain at pH 3.0.

All strains grew very slowly at low pHs , this trend was previously reported by Lankaputhra *et al.*, (1996) who

found that bifidobacterial viability was seriously affected by any drop in pH below 4.3. Also , the effect of low pH on the growth of bifidobacteria was investigated by Sakai *et al.*, (1987) who found that low pH was important determinant in bifidobacterial mortality .

Low pH had an effect as increasing undissociated acids levels , which was more harmful to microorganisms and was clearly a factor in the rapidly decreasing bifidobacterial population (Adams and Moss, 2000).

Data in Fig (1) revealed that some isolates tolerated greatly pH 4.0 for as long as 48 hrs. Bao *et al.*, (2009) confirmed the good tolerance of probiotic isolates to low pH levels. Hoque *et al.*, (2010) reported that many probiotics were tolerant to pH 3.0 or lower. Boke *et al.*, (2010) explained the resistance to low pH to be due to the exopolysaccharides (EPSS) production by probiotics. They also added that the high EPSS producing strains showed a significant protective effect against low pH (2.0).

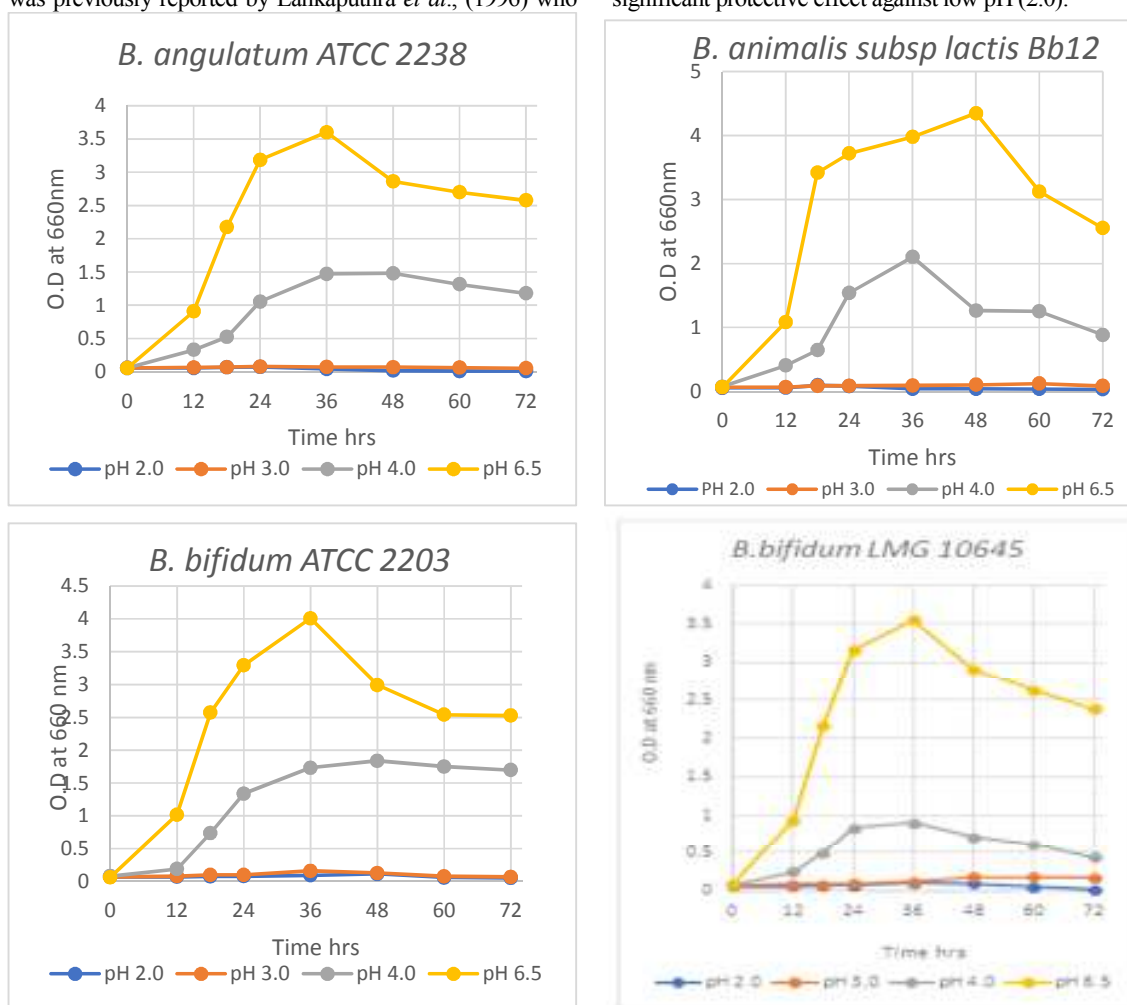


Fig. 1. The impact of different pHs on the growth of bifidobacteria at 37°C for 72 hrs

Fig (2) shows the ability of the strains to decrease the pH of the MRS by producing acids , *Bifidobacterium animalis subsp lactis Bb12* the most active one at a wide range of pH ranging from pH 3.0 - pH 6.5 . All strains could not reduce much the pH of the medium at pH 2.0 .

Humans secrete approximately 2.5 liters of gastric juice every day, generating a fasting gastric pH

of 1.5, which increases to between pH 3.0 and 5.0 during feeding (Hill,2002). In order to survive in the intestinal tract, a probiotic candidate should tolerate or be resistant to gastric acid (HCl) for at least 90 minutes (Chou and Weimer, 1999).

In addition to processing foods to minimize bacterial survival, the opposite can be desirable. Foods,

especially those with a good buffering capacity, are frequently used as carriers for probiotic bacteria to ensure survival and passage through the acidic stomach. Furthermore , acid adaptation could greatly aid the

survival of these cultures not only in the stomach but also against other stresses subsequently encountered in the gastrointestinal tract (Cotter and Hill ,2003).

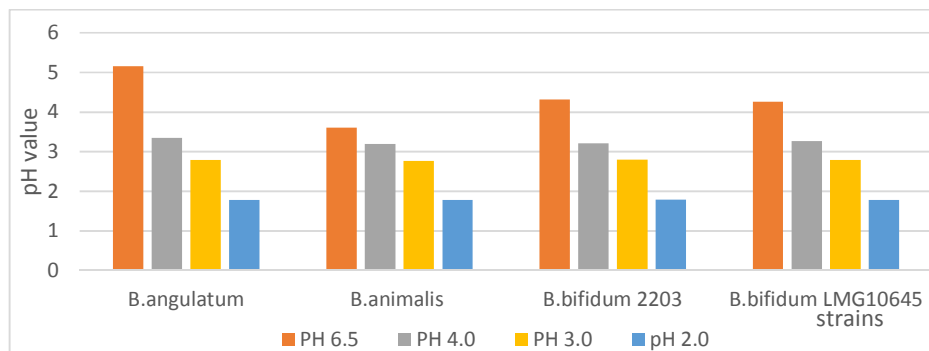


Fig. 2. pH values of the bifidobacterial strains after 72 hrs of incubation in MRS+L.cys medium

2- Growth of bifidobacteria in different types of milk
Human milk

Bifidobacterium angulatum 2238, *Bifidobacterium bifidum* 2203, *Bifidobacterium animalis subsp lactis* Bb12 and *Bifidobacterium bifidum* LMG 10645 grew in human milk at the rate of 1.72×10^7 , 7.5×10^6 , 2.6×10^7 and 5.9×10^6 respectively, Table (1) . Ročkova *et al.*, (2013) reported that human milk was less effective for growing two strains of *Bifidobacterium animalis subsp lactis* isolated from fermented dairy product in comparison with cow , goat and sheep milk that effect was due to the high concentration of antimicrobial systems in human milk such as milk (lactoferrin, haptocorrin, immunoglobulins, lactoperoxidase, and lysozyme) . Clare *et al.*, (2003), found that the lysozyme concentration in Cow’s and goat’s milks are very

low (0.16 and 0.23 mg/l) relative to human milk (400 µg/ml) and recently study by Ročkova *et al.*, (2013) revealed that lysozyme content in human ,cow and sheep milk was $27.91 < 1$ and < 1 respectively . These data may explain the incidence drop in the bifidobacterial growth especially in the two *B. bifidum* in human milk compared to cow , goat and sheep milk .

Moreover , the ability of oligosaccharides utilization is strain dependent , Zivkovic and Barile (2011) reported that only *Bifidobacterium longum ssp. infantis* (*B. infantis*), a *bifidobacterium* enriched in the gastrointestinal tracts of healthy breast-fed infants has a unique gene cassette that allows it to transport and metabolize the specific oligosaccharide structures found in human milk .

Table 1. growth of four strains of bifidobacteria in different types of milk at 37°C for 24 hrs .

milk type strain	Viable bacterial number (cfu /ml)					
	Human	Cow	Goat	Buffalo	Sheep	Camel
<i>Bifidobacterium angulatum</i> ATCC 2238	1.72×10^7	2.1×10^7	1.7×10^6	1.08×10^7	1.5×10^6	2.83×10^6
<i>Bifidobacterium bifidum</i> ATCC 2203	7.5×10^6	4.5×10^7	10×10^9	3.4×10^7	1.11×10^8	1.62×10^7
<i>Bifidobacterium animalis subsp lactis</i> Bb12	2.6×10^7	2.59×10^7	1.12×10^8	5×10^6	5.5×10^7	7.5×10^5
<i>Bifidobacterium bifidum</i> LMG 10645	5.9×10^6	2.6×10^7	3.3×10^7	2.04×10^7	2.5×10^7	1.15×10^7

Results are expressed as mean values of three replicants .

Cow milk

Table (1) showed that all of the studied strains grow nearly at the same rate in bovine milk 10^7 cfu/ml. However, bovine milk has a much lower concentration of oligosaccharides than human milk, and the majority of the molecules are simpler in structure than those found in human milk so, bovine milk could stimulate *Bifidobacterium* species growth .Specific structural characteristics of milk-derived oligosaccharides are crucial to their ability to selectively enrich beneficial bacteria while inhibiting or being less than ideal substrates for undesirable and pathogenic bacteria (Zivkovic and Barile.,2011).

Ročkova *et al.*, (2013) reported that cow milk can be used as a carrier for bifidobacteria that encourage its use in industrial scale. Whey proteins such as α lactalbumine and β -lactoglobulin were found to be excellent growth medium for bifidobacteria (Pestschow and Talbott, 1990). Other biological compounds identified as growth factor for bifidobacteria include threonine and cysteine (Roy *et al.*, 1990).

Ceballos *et al.*, (2009) reported that pH values of cow milk were higher than those of goat milk after pasteurization and before the inoculation of the starter culture . The amounts of fatty acids C6:C10 are at least two-fold higher in goat’s milk than in cow’s milk . According to Chilliard *et al.*,(2006), the differences between the long-chain fatty acids levels in the fat of goat’s and cow’s milk may be due to the differences in ruminal adipose tissue metabolism between the two animal species.

Goat milk

Bifidobacterium animalis subsp lactis Bb12, *Bifidobacterium bifidum* 2203 and *Bifidobacterium bifidum* LMG 10645 exert their best growth at goat milk 10×10^9 cfu/ml , 1.12×10^8 cfu/ml and 3.3×10^7 cfu/ml,. Two strains of human origin *B. bifidum* 1, 2 can grow in goat’s milk, producing 1235 mg/L of lactic acid at pH 5.0 Ročkova *et al.*, (2013).

The better growth in goat milk may due to goat milk content of lactose-derived oligosaccharides and conjugated linoleic acid which is an isomer of linoleic acid with no inhibition effect on bifidobacteria . Also , some species of

bifidobacterial convert linoleic acid to conjugated-linoleic acid as a mechanism of detoxification of linoleic acid and higher vitamin (A and complex B) (Adamczak et al., 2008 and Al-Abdulkarim et al., 2013) in addition to its low content of lysozyme of lysozyme (0.23 mg/l) reported by Clare et al., (2003).

Buffalo milk

Bifidobacterium angulatum 2238 , *Bifidobacterium bifidum* 2203, *Bifidobacterium animalis subsp lactis Bb12* and *Bifidobacterium bifidum* LMG 10645 grew in buffalo milk as follow 1.08×10^7 cfu /ml, 3.4×10^7 cfu /ml, 5×10^6 cfu /ml and 2.04×10^7 cfu /ml respectively. Table (1) , Jayamanne and Adams, (2004) observed that Bifidobacteria survived for 3 days above the required population level of 10^6 CFU /g) in buffalo curd . High fat in buffalo milk has an inhibitory effect on the bacterial growth vise versa the low fat buffalo milk is a good vehicle for probiotics (Han , 2012).

Maintaining the viability of bifidobacterial in buffalo milk and fermented dairy foods requires a low redox potential since excess oxygen leads to increased production of harmful reactive oxygen species (Lankaputhra et al., 1996).

Sheep milk

Sheep milk stimulate the growth of bifidobacterial strains especially *Bifidobacterium bifidum* 2203 and *Bifidobacterium animalis subsp lactis Bb12*. *B. animalis subsp. Lactis* was 5.5×10^7 this result is in agreement with Ročková et al., (2013) who reported that *B. animalis subsp. lactis* which slightly grew in sheep's milk . It was found that although the sheep milk has higher protein and vitamin content but it was not sufficient to sustain growth of *B. lactis* at the high rate required (Kurmann, 1998).

Bifidobacterium bifidum, is a slow-growing bacteria as a single species in milk due to its weak proteolysis activity and lack of nonprotein nitrogen (NPN) in milk that make milk into an unsuitable environment for growth of *B. bifidum*. (Marhamatizadeh et al., 2012).

Camel milk

Results in Table (1) showed the bifidobacterial count in camel milk . It was observed that viable bacterial number for *Bifidobacterium animalis subsp lactis Bb12* was found to be the lowest followed by *Bifidobacterium angulatum* 2238 . Ramet, (2001) found that the buffering capacity was high in camel milk compared to cow milk . The increasing buffering capacity in camel milk lead to stabilize the pH, which encourages the growth of bifidobacterial (Shori and Baba, 2011). It was observed that the obtained data in Table (1) were contradictory . Camel milk was reported to have a high antimicrobial lactoperoxidase system (Anonymous, 2003) which may be caused by further inhibitory effects on bifidobacteria strains particularly *Bifidobacterium animalis subsp lactis Bb12* that reached 7.5×10^5 cfu/mL after 24 hrs of incubation .The present data are in agreement with studies obtained by (Ranadheera et al., 2012). Similar rate 13.2×10^6 cfu/mL of viable cell count of *B. bifidum* in camel-milk was recorded by (Shori and Baba , 2014)

It could be concluded that all of the studied strains tolerated low PH values and can grow at the selected mammalian milk except camel milk that appears to be

quite suitable for the survival of the minimum population level for an acceptable probiotic product.

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تحمل الحموضه ونمو اربعة سلالات من البيفيدوبكتريا في انواع مختلفه من الالبان رغد مختار سيد معوض ، صباح توني عبدالرازق ، احمد شوقي زهران و فوزي سيد ابراهيم قسم علوم الالبان – كلية الزراعة – جامعه المنيا

في هذه الدراسة تم دراسة مقدرة اربعة سلالات من البيفيدوبكتريا علي تحمل مدي واسع من الرقم الهيدروجيني (pH) فقد تراوح بين (6.5 - 2.0) ووجد أن السلالات بنفس المعدل تقريبا علي درجات 2.0 و 3.0 من رقم الاس الهيدروجيني. كما درست مقدرة السلالات علي خفض رقم الاس الهيدروجيني بانتاج الحموضه وكانت السلالة *Bifidobacterium animalis subsp lactis* هي الاعلي في مقدرتها علي خفض رقم الاس الهيدروجيني علي درجات 3.0 , 4.0 و 6.5 بينما اظهرت السلالات نفس المعدل علي رقم هيدروجيني 2.0 حيث خفض الرقم من 2.0 الي 1.80 . وقد تناولت الدراسة ايضا مقارنه بين السلالات في المقدرة علي النمو في انواع مختلفه من الالبان وهي لبن الأم ، البقري ، الجاموسي ، الماعز ، الاغنام و لبن الابل . واستطاعت السلالات النمو بمعدلات تتناسب و الحد الأدنى لاحداث التأثيرات الصحيه لهذه البكتريا الحيويه وهو من 10^7 - 10^6 باستثناء لبن الابل حيث نمت بعض السلالات البكتيرييه به بمعدل اقل وهو 7.5×10^5 .