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ISOLATION AND CHARACTERIZATION OF BACTERIA WITH STRONG PROBIOTIC CRITERIA

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ABSTRACT

A well-known traditional dairy product in the rural areas in Upper Egypt, Laban zeer was used as a natural source to isolate bacteria with probiotic criteria. Investigations for probiotic criteria of the obtained bacterial isolates were conducted in order experiments, in which the unsuitable isolates were discarded. With the exception of yeast isolates, which were excluded, eleven isolates (7 bacilli and 4 cocci) were obtained and purified. These isolates were found to be Gram positive, catalase negative, non-spore-formers and non-motile, which meets the basic characteristics of some probiotic bacteria. These experiments resulted in the selection of three isolates (2 bacilli and 1 cocci) with high acid and bile salt tolerance since they grew well in media with pH 3 and contained bile salt at concentration up to 0.3%. In addition, the isolated bacilli and cocci showed strong adhesion to epithelial cells of sheep intestine.
Keywords: probiotic, Laban Zeer, acid and bile salt tolerance, adhesion.

INTRODUCTION

According to Lilly and Stilwell as early as the year 1965, the term "probiotic" originally referred to microorganisms that have effects on other microorganisms. Fuller in 1989 described probiotics as a "live microbial feed supplement which beneficially affects the host animal by improving its Intestinal microbial balance". The term probiotics was taken up by Tannock, (2003) who defined the concept as, “organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal microbial balance”. In the following decades,

intestinal lactic acid bacterial species with alleged health beneficial properties have been introduced as probiotics, including *Lactobacillus rhamnosus*, *Lactobacillus casei*, and *Lactobacillus johnsonii* (Gueimonde and Collado, 2012).

In Egypt, specific probiotic strains with documented health benefits, Ali *et al.,* (2013A) are sparsely available not affordable to the majority of the population. Suitable strains need to be chosen and efforts are needed to make products as cereal or milk foods could make a profound impact on the health and well-being of adults and children. This can gauge the impact of probiotics on consumers' nutrition and health, and increase the number of people who can benefit. This illustrates the necessity for isolation bacteria from natural source with strong and stable probiotic criteria to be used for commercial purpose, Ali *et al.,* (2013B).

The aim of the present work is to isolate probiotic bacteria from zeer milk on the MRS broth (DeMan *et al.,* 1960) screen them for their ability to grow and to tolerate the effect of low pH and bile salt and the intensity of adhesion cells lining theintestine sheep

2. MATERIALS AND METHODS

2. 1. Isolation

Laban Zeer collected from a rural area in Minia Governorate was used as a natural source for the desired bacteria. The samples of Laban Zeer were serially diluted in sterilized water. Growth of bacterial isolates was performed by growing in MRS agar at 37 °C for 48 hrs. Volumes of 0.1 ml from the appropriate dilution were plated on MRS agar plates. After 48 hrs the growing colonies were picked up and purified by streaking on MRS agar plates.

2. 2. Assay for morphology, Gram stain and catalase test as probiotic properties:

Tests for probiotic properties of the obtained bacterial isolates were conducted in order steps, in which any strain when showing good performance for a tested property was again selected for the next tests, and the unsuitable isolates were discarded. Therefore, the obtained isolates were tested primarily for probiotic proprieties by: microscopic examination for their morphology, Gram stain and catalase production (Klayraung *et al.,* 2008 and Al-Awwad *et al.,* 2009). The Gram positive and catalase negative bacteria (probiotics properties) were then selected for spore forming and motility tests. The spore forming, motile bacteria were discarded.

2. 3. Screening the bacterial isolates for Acid and Bile salt tolerance as probiotic properties:

The isolates showed Gram positive and catalse negative reactions were again tested for acid and bile tolerance. Three successive experiments were carried out in this respect as follow:-

2. 3. 1. Acid tolerance

This experiment was conducted in test tubes, each containing 10 ml MRS

broth. The tubes were classified into four groups, one group was adjusted to pH 7 (as control) and the other three groups were adjusted to pHs 6, 5, and 4 by using 0.1 N HCL, and then sterilized by autoclaving at 121 ^{o}C for 15 min. Active cultures of each bacterial isolates were inoculated (1%v/v) into pH adjusted broth. The test tubes were incubated at 37 ^{o}C for 48 hrs. Each treatment was replicated 3 times (in 3 test tubes). Immediately after inoculation (zero time), and after 48 hrs of incubation, the acid tolerance was determined by measuring the bacterial growth in the treated tubes. This growth was monitored by measuring the number of viable cells in treated tubes. The isolates which were highly tolerant to acidity and showed high growth at acid concentration up to pH 4 selected and subjected to assay for bile salt tolerance.

2. 3. 2. Bile tolerance

The isolates showed good acid tolerance were tested for bile tolerance which conducted according to the method described by Walker and Gilliland, (1993) and Al-Saleh *et al.*, (1998). It was almost similar to the previous test. All cultures were evaluated for their growth at 37 ^{o}C for 48 hrs in MRS broth provided with 0.0, 0.1, 0.2, and 0.3% (w/v) bile salt (oxgall).

2. 4. Test of bacterial adhesion to intestinal epithelial cells as a probiotic property:

Adhesion of bacterial isolates to columnar epithelial cells of sheep was tested using the method of Fuller, (1973) and Bekheet, (2008). The test isolate cultures (each in 10 ml MRS broth) were centrifuged and the pellets were re-suspended each in 10 ml of buffer saline (pH 7.2). The crop scrapings were prepared by removing the organ, opening with scissors and washing in buffer. The epithelium layer was then scrapped off with the edge of a microscope slide and the scrapings were re-suspended in the buffer. An amount of 0.1 ml bacterial cells suspension was added to 0.4 ml of the epithelial suspension. The mixture was rotated for 30 min at 37 °C. Adhesion was examined by light microscopy of Gram stained preparations, and pictures of adhesion were prepared.

3. RESULTS AND DISCUSSION

3. 1. Isolation of bacteria with probiotic properties

Since it well known that most of the probiotic bacteria are members in the family Lactobacillaceae, this work aimed to isolate probiotics from the natural source of lactobacillaceae. Eleven strains were isolated from Laban Zeer as shown in Table (1). These isolates were recovered from MRS agar and were purified. The morphological study showed that some isolates were yeasts. The isolates looks like yeasts were excluded since they are not considered as probiotics yet. The other 11 isolates were found to be Gram

positive, catalase negative, non-spore-formers and non-motile, which meets the basic probiotic characteristics. Those 11 isolates included 7 rod-shaped and 4 cocci-shaped isolates, and they were retained for further assessment for potential probiotic properties. In earlier studies, fermented milk and other dairy products have been reported as a major source of probiotic lactic acid bacteria by several authors (Ali *et al.*, 2010). For example Bukola and Abiodun (2008) isolated and characterized one hundred and fifteen strains of lactic acid bacteria from some fermented dairy ("Nono", "Fura", Yogurt, "Wara") and non-dairy foods ("Ogi" and "Fufu").

3. 2. Cod of isolates:

For convenient discussion the isolates were given cods as follow:

L1Lz = *Lactobacillus* No. 1 from Laban zeer, L2Lz = *Lactobacillus* No, 2 from Laban zeer and so on.

S1Lz = *Streptococcus* No. 1 from Laban zeer, S2Lz = *Streptococcus* No. 2 from Laban zeer and so on.

Table (1): Description of the obtained isolates from Laban zeer.

Isolate	Description	Genus
L1Lz	Rod shaped Gram positive, catalase negative, non-spore-formers and non-motile	*Lactobacillus*
L2Lz	Rod shaped Gram positive, catalase negative, non-spore-formers and non-motile	*Lactobacillus*
L3Lz	Rod shaped Gram positive, catalase negative, non-spore-formers and non-motile	*Lactobacillus*
L4Lz	Rod shaped Gram positive, catalase negative, non-spore-formers and non-motile	*Lactobacillus*
L5Lz	Rod shaped Gram positive, catalase negative, non-spore-formers and non-motile	*Lactobacillus*
L6Lz	Rod shaped Gram positive, catalase negative, non-spore-formers and non-motile	*Lactobacillus*
L7Lz	Rod shaped Gram positive, catalase negative, non-spore-formers and non-motile	*Lactobacillus*
S1Lz	Coccus shaped Gram positive, catalase negative, non-spore-formers and non-motile	*Streptococcus*
S2Lz	Coccus shaped Gram positive, catalase negative, non-spore-formers and non-motile	*Streptococcus*
S3Lz	Coccus shaped Gram positive, catalase negative, non-spore-formers and non-motile	*Streptococcus*
S4Lz	Coccus shaped Gram positive, catalase negative, non-spore-formers and non-motile	*Streptococcus*

3. 3. Acid tolerance

The isolates showed Gram positive, catalase negative reactions and rod or cocci shaped were again tested for acid tolerance. This experiment was conducted in test tubes each containing 10 ml MRS broth adjusted to different pH values as indicated above in material and methods section. The results of this experiment are illustrated in Table (2). These results showed that all tested isolates were able to grow well at pH values of 7 and 6 with no significant difference. At pH 5 the growth of 4 isolates dropped sharply by more than 60% as compared with the growth of these isolates at pH of 7 and 6. Furthermore the growth of these 4 isolates completely inhibited at pH 4. On the other hand the rest of the studied isolates (7 isolates) showed excellent growth at pH 5 without any clear difference in the growth as compared with the situations at pH 7 and pH 6. Furthermore these 7 isolates showed good growth at pH 4. Accordingly, the 4 isolates showed a weak growth at pH 5 and no growth at pH 4 were excluded and were considered as not probiotic bacteria. However there was a probability that the other 7 isolates, who showed good tolerance to acidity are probiotic bacteria. Therefore the decision was to continue screening these isolates and select the most efficient isolates via testing them for other probiotic criteria. It is well known that tolerance to acidity is one of the most important probiotic criteria. 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). The obtained data illustrated in Table (2) revealed that 7 isolates tolerated greatly pH 4 for a period of 48 hrs. In previous studies, several reports confirmed the good tolerance of probiotic isolates mainly lactobacilli to low pH levels (Succi *et al.,* 2005; Harutoshi *et al.,* 2007; Bao *et al.,* 2009 and Ali *et al.,* 2010). Boke *et al.,* (2010) explained the resistance to low pH to be due to the exopolysaccharides (EPSS) production by probiotics. He added that the high EPSS producing strains showed a significant protective effect against low pH (2.0).

3. 4. Bile salt tolerance

Out of the 11 bacterial isolates previously tested for acid tolerance (down to pH 4), 7 isolates were selected, based on their high acid tolerance, to be evaluated for bile salt tolerance and the results are given in Tables (3). The results showed that all of these 7 bacterial isolates grew well at bile salt concentration of 0.1% since there were no significant differences in their growth at 0.1% and 0.0% bile salt concentration. As shown the growth of two isolates sharply decreased at bile salt concentration of 0.2% and completely inhibited at concentration of 0.3%. These two isolates were excluded since they considered not probiotic bacteria.

On the other hand, the results showed that 5 isolates from the tested 7 isolates were able to grow well in media contained bile salt concentration up to 0.3%. These 5 isolates were considered for the next experiments. As shown in Table (3) these 5 isolates consisted of 4 isolates lactobacilli and only one isolate streptococci.

Table (2): Tolerance of studied isolates to acidity.

Isolate No.	Growth of the studied isolates at different pH values as number ($\times 10^6$)/ml of liquid culture			
	pH 7	pH 6	pH 5	pH 4
L1Lz	149	143	132	90
L2Lz	115	110	37	00
L3Lz	120	100	100	80
L4Lz	115	113	85	80
L5Lz	120	110	34	00
L6Lz	140	142	139	92
L7Lz	151	150	93	90
S1Lz	151	148	30	00
S2Lz	144	143	32	00
S3Lz	138	138	131	91
S4Lz	144	145	100	90

Table (3): Tolerance of studied isolates to bile salt at concentration up to 0.3%

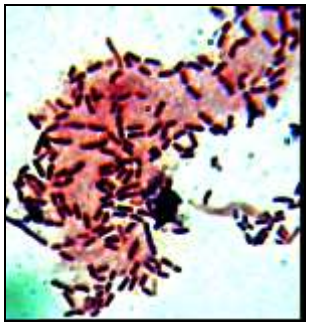
Isolate	Growth of the studied isolates at different bile salt concentration as number ($\times 10^6$)/ml of liquid culture			
	Bile salt concentration (%)			
	0.0	0.1	0.2	0.3
L1Lz	143	131	119	105
L3Lz	134	123	98	80
L4Lz	122	89	35	0.0
L6Lz	144	144	112	97
L7Lz	133	133	111	99
S3Lz	129	122	118	102
S4Lz	144	100	38	0.0

Goldin and Gorbach (1992) mentioned that concentrations of 0.15 – 0.3% of bile salts have been recommended as suitable for selecting probiotic bacteria for human use. The release of bile salts hydrolase (BSH)

enzyme by certain bacteria was given as the factor responsible for the mechanism of resistance of probiotics to bile salts. This enzyme had been detected in the gut microflora genera such as *Lactobacillus* and *Bifidobacterium* (Tanaka *et al.,* 1999). In addition to the role of the BSH enzyme in bile tolerance, Boke et al. (2010) also reported that the production of exopolysaccharides (EPSS) by certain strains showed a significant protective effect against bile salts. In agreement with the present results, Thirabunyanon et al. (2009) and Hoque *et al.,* (2010) tested the same concentration of bile salts (0.3%) against their dairy products isolates to prove their potential probiotic activity.

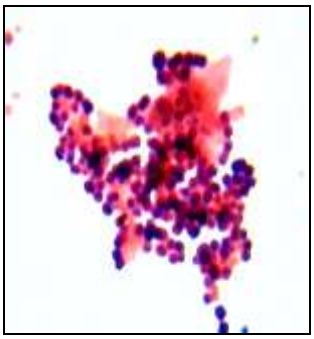
3. 5. Adhesion to Sheep Intestinal Epithelial Cells:

Adherence to intestine epithelial tissues is considered important criteria for the selection of probiotic bacteria. The results of the adhesion ability test (in vitro) of a chosen 5 bacterial isolates (4 rod-shaped, and 1 cocci-shaped) to columnar epithelial cells of sheep intestine were done, (Ali *et al.,* 2010). Adhesion examined by light microscopy of Gram stained preparations, revealed that out of the tested 5 isolates, 2 showed no adhesion to sheep intestinal epithelial cells and the other 3 isolates (2 rod-shaped, and 1 cocci-shaped) showed strong adhesion as shown in Fig. 1.



L1Lz

L3Lz



S3Lz

Fig. 1 Pictures of adhesion of the selected isolates as appeared under the light microscopy

Earlier, Dunne *et al.,* (2001) reported that the adhesion to gut epithelial tissue (Intestinal mucosal cells) and the ability to colonize the gastrointestinal tract are considered important criteria for the selection of probiotic bacteria. In agreement with the obtained results, Fuller *et al.,* (1978) reported the adherence of *Lactobacillus* and *Streptococcus* to epithelial cells in vitro. In addition, *Lactobacillus rhamnosus* was shown to adhere in vitro to BMM cells line and in vivo to intestinal epithelial cells of chicken and therefore may be considered as a potential probiotic for chicken (Bouzaine et al. 2005). The adhesion of *Lactobacillus* to Caco-2 cells line of intestine have also been reported by Ronka *et al.,* 2003; and Gueimonde *et al.,* 2006;. Miyoshi et al. (2006) also reported that the mechanism of *Lactobacillus reuteri* adhesion in the gastrointestinal tract appeared due to the binding of MapA (a surface protein of the bacterium) to receptor-like molecules on Caco-2 cells in the intestinal tract.

REFERENCES

Al-Awwad , N.J., Haddadin, M. S. and Takruri, H.R. (2009): The characteristics of locally isolated *Lactobacillus acidophilus* and *Bifidobacterium infantis* isolates as probiotics Strains. Jordan J. Agric. Sci., 5 (.2): 192-206.

Ali, F. S., Saad, O. A . and Salwa A.G.,Hussein(2010).The production of probiotic yougurt. I.Isolation and Screening of probiotic bacteri.The 5th Arab Conference for Food Technology and Dairy Sciences. October 18-22. Mansoura/Luxure,Mansoura University, Egypt.

Ali, F. S., Saad, O. A. O. and Salwa, A .G.. Hussein (2013A). Antimicrobial activity of probiotic bacteria.The second Internationag Conference of Biological Sciences(The Role of Biological Sciences in The Development of Society).Egypt – Cairo 1-3 July 2013.

Ali, F. S., Saad, O. A.O and Salwa, A .G. Hussein (2013B), Probiotic stability of yoghurts during refrigerated storage. The second Internationag Conference of Biological Sciences(The Role of Biological Sciences in The Development of Society).Egypt – Cairo 1-3 July 2013.

Al-Saleh, A.A., Zahran, A.S. and Abu-Tarboush, H.M. (1998): Growth of bifidobacteria: environmental conditions and adherence to epithelial ceils. Milchwissen-schaft 53 (4) 187-190.

Bao, Y. , Zhang , Ya. Zhang, Yo. Liu, Y. Wanga, S. Dong , X. Wang, Y. and Zhang, H. (2009): Screening of potential probiotic properties of *Lactobacillus fermentum* isolated

from traditional dairy products. J. Food Cont.,21: 695-701.

Bekheet, M.M.E. (2008) Growth and activity of lactic acid bacteria in soymilk. M.S. Thesis, Fac. Agric., Minia Univ., Egypt, pp 142.

Boke, H., B. Aslim and G. Alp (2010): The role of resistance to bile salts and acid tolerance of exopolysaccharides (EPSS) produced by yoghurt starter bacteria. Arch. Biol. Sci., Belgrade, 62 (2), 323-328.

Bouzaine, T., Dauphin, R.D. Thonart, P.. Urdaci, M.C and Hamdi, M. (2005): Adherence and colonization properties of *Lactobacillus rhamnosus* TB1, a broiler chicken isolate. Lett. Appl. Microbiol., 40: 391 – 396.

Bukola, C.A. and Abiodun, A. O. (2008). Screening of Lactic Acid Bacteria Strains Isolated from Some Nigerian Fermented Foods for Exopolysaccharides Production. *World Applied Sciences Journal* 4 (5): 741-747.

Chou, L. S. and Weimer, B. (1999): Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. J. Dairy Sci. 82 (1):23–31.

DeMan, J.D., Rogosa; M.A. and Sharpe, M.E. (1960): A medium for the cultivation of lactobacilli. J. Appl. Bact., 23: 130-135.

Dunne, C.L.; O'Mahony, L.M.; Thornton, G.; Morrissey, D.; O'Halloran, S.; Feeney, M.; Flynn, S.; Fitzgerald, G.; Daly, C.; Kiely, B.; O'Sullivan, G.; Shanahan, F. and Collins, J.K. (2001). In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. Am. J. Clin. Nutr., 73(2) 386 – 392.

Fuller, R. (1973): Ecological studies of *Lactobacillus* flora associated with the crop epithelium of the fowl. J. Appl. Bacteriol. 38:131-139.Fuller, R. (1973): Ecological studies of *Lactobacillus* flora associated with the crop epithelium of the fowl. J. Appl. Bacteriol. 38:131-139.

Fuller, R. (1989) Probiotics in man and animals. J. Appl. Bacteriol.; 66: 365–78.

Fuller, R., Barrow, P. A. and Brooker, B. E (1978): Bacteria associated with the gastric epithelium of neonatal pigs. Appl. Environ. Microbiol. 35(3): 582-591

Goldin, B.R. and Gorbach, S.L. (1992): probiotic for humans. In Fuller, R. (Ed.), probiotics, the scientific basis. Chapman and hall, London, pp. 355 – 376.

Gueimonde, M., Jalonen, L. He, F. Hiramatsu, M. and Salminen, S. (2006): Adhesion and competitive inhibition and displacement of human entero-pathogens by selected lactobacilli. Food Res. Int., 39 (4): 467-471.

Gueimonde, M. and Collado, M.C.(2012) Metagenomics and probiotics. Clinical Microbiology and Infection. 18: 32-34.

Harutoshi, T., Kazushi, H. and Taku, M. (2007): High bile- and low pH-resistant lactic acid bacteria isolated from traditional fermented dairy products in Inner Mongolia, China **.** Milk Sci.55 (3**)**:129-134.

Hoque, M.;ZAkter, F.; Hossain, K.M.; Rahman, M.S.M.; Billah, M.M. and Islam, K.M.D. (2010): Isolation, identification and analysis of probiotic properties of *Lactobacillus spp.* from selective regional yoghurts. World J. Dairy & Food Sci. 5 (1): 39-46.

Klayraung , S., Viernstein , H. Sirithunyalug, J. and Okonogi, S. (2008): Probiotic Properties of Lactobacilli Isolated from Thai Traditional Food. Sci. Pharm. 76: 485–503.

Lilly, D. M. and Stillwell, R. H.. (1965) Probiotics: growth promoting factors produced by microorganisms. Science 147:747-748.

Miyoshi, Y. , Okada, S. Uchimura, T. and Satoh, E. (2006): A mucus adhesion promoting protein, mapA, mediates the adhesion of *Lactobacillus reuteri* to Caco-2 human intestinal epithelial cells. Biosci. Biotechnol. Biochem., 70 (7), 1622-1628.

Ronka, E., Malinena, E.. Saarelab, M. Rinta-Koskic, M Aarnikunnasa, J. and Palva, A. (2003): Probiotic and milk technological properties of *Lactobacillus brevis.* Inter. J. Food Microbiol., 83:63-74.

Succi, M., Tremonte , P. Reale , A. Grazia , E.. Pacifico, S and Coppola, R. (2005): Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. FEMS Microbiol. Lett. 244 (1):129-137.

Tanaka, H..; Doesberg, K.; Iwasaki T. and Mierau, I. (1999): Screening of lctic acid bacteria for bile salts hydrolase activity. J. Dairy Sci., 82: 2530 – 2535.

Tannock, R. (2003). Neuropsychology of attention disorders. In S. J. Segalowitz & I. Rapin (Eds.), Handbook of neuropsychology (2nd ed.). Netherlands: Elsevier Science

Thirabunyanon, M., Boonprasom, P and Niamsup, P. n (2009) : Probiotic potential of lactic acid bacteria isolated from fermented dairy milks on antiproliferation of colon cancer cells. Biotechnol. Lett., 31: 571-576.

Walker, D.K. and Gilliland, S.E (1993): Relationship among bile tolerance, bile salt deconjugation, and assimilation of cholesterol by *Lactobacillus acidophilus*. J. Dairy Sci., 76(4):956-61.

الملخص العربي

عزل وتوصيف بكتيريا لها معايير بروبيوتك قوية

فاروق شحاتة علي، جابر زايد بريشة، محمد زكي حسن، مها عمر عبداللطيف عمر
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تم استخدام لبن الزير وهو أحد منتجات الألبان التقليدية المعروفة جيدا في المناطق الريفية في صعيد مصر ، كمصدر طبيعي لعزل بكتيريا تنطبق عليها معايير بكتيريا البروبيوتيك . باستثناء عزلات الخميرة، التي استبعدت ، تم الحصول على أحد عشر عزله (7 عصويات و 4 كرويات) وتم تنقيتها. وقد أوضحت التجارب أن هذه العزلات موجبة لصبغة جرام، وسالبة لاختبار الكتاليز، وغير متجرثمة، وغير متحركة، وهذه الصفات تعتبر أساسية في بعض أجناس بكتيريا البروبيوتيك المعروفه . ثم أجريت اختبارات معايير اختبار البكتيريا تتبع بكتيريا البروبيوتيك على العزلات البكتيرية التي تم الحصول عليها في سلسلة من التجارب بحيث يتم في كل تجربة استبعاد العزلات التي لا ينطبق عليها المعيار محل الدراسة. وفي نهاية هذه التجارب تم الحصول على ثلاث عزلات (اثنين عصويات وواحدة كروية). هذه العزلات الثلاث تميزت بتحمل حموضة عالية وتحمل تركيزات عالية من أملاح الصفراء، حيث نمت جيداً في بيئات غذائية لها رقم أس هيدروجيني (pH) 3 ونمت أيضاً جيداً في بيئات عذائية بها تركيز عالي من ملح الصفراء يصل إلى 0.3 ٪ . وبالإضافة إلى ذلك، أظهرت الثلاث عزلات المتحصل عليها التصاق قوي للخلايا المخاطية المبطنة لأمعاء الأغنام.

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