Vet.Med.J.,Giza. Vol.53, No.2. (2005): 241-249.

IMPORTANCE OF BACTERIOCIN OF LACTOBACILLUS ACIDOPHILUS IN PREVENTION , COLONIZATION AND PROLIFERATION OF ENTERIC BACTERIA IN CATTLE

SAHAR . R . MOHAMED and AZZA . N. FARAG
Bacteriology Department Animal Health Research Institute
Doki, Cairo, Egypt

Received: 20.3.2005.
Accepted: 27.3.2005.

SUMMARY

Bacteriocin produced by Lactobacillus acidophilus was tested for it`s potential as aprobiotic culture, it`s exhibited good sensitivity to heat, protease enzyme, acid tolerance, as well as bile resistance in media containing 0.3% bile acids.

Bacteriocin was active over wide pH range and inhibted a number of pathogenic bacteria.The SDS - PAGE of the active fraction resulted in a single band with molecular mass of 5.4 KDa. In challenge test. Mice that were fed on milk based diets contain bacteriocin (10^8 C.F.U).showed 100%. protection level . Meanwhile the antibody titers in the serum of immunized mice with bacteriocin were significantly higher than the titers in the control of unprotected mice . These results confirmed the probiotic effect of bacteriocin against colonization of most microorganism in the animal tissue as well as enhancing their immune response.

INTRODUCTION

Intstinal lactic acid bacteria for animal are closely associated with the host health because lactic acid is an important biodefense factor in preventing colonization and subsequent proliferation of pathogenic bacteria (Gilliland, 1986).

A probiotic is defined as a live microbial. feed supplement which beneficially affects the host animal by improving its intestinal microbial balance. (Kalchayanand et al., 1992).

Lactic acid have been claimed as probiotics as Lactobacillus acidophlus this bacteria should become apart of the normal microbial flora, survive the gastrointestinal, passage and able to adhere and colonize (R) (Shu and Gill 2002).

The gastrointestinal tract of healthy animals contain gastric juices, digestive enzyme and bile ac-

ids . These conditions impose a significant threat to probiotic strains.

In addition immune response. Affect ther survival of probiotic strains (Jack et al ., 1998).

Since many pathogenic bacteria of different varieties inhibit the intestine and its challenged for probistic straint to become established as gasterio-intestinal microflora, thus organisms that can produce a product that will inhibit the growth or kill existing organisms in intestine (Hudault et al 1997).Lactic acid bacteria produce a number of antimicrobial substance, icluding. Bacteriocins which are peptides exhibit inhibitory activity against sensitive strains of bacteria (Tahara et al., 1992 , Jack , et al., 1995).

Bacteriocins that are produces by strains of *L..acidophilus* have been purified and characterized (Zamifir et al., 1999) .

The purpose of this study was to evaluate the bacterioum as acid and bile tolerence and act as protective immune response when challenged mice by pathogenic microorganism.

MATERIAL AND METHODS

Bacterial strains:

Pathogenic bacteria were incubated in trypticase soya broth at 37cfor 18 to 24 h, before use.

Lactic acid bacteria were grown for 18 h at 37c in MRS broth .

Bacteriocin production and activity (Toba etal 1991)

Bcteria Cell were removed from the growth medium by centrifugation (6.000 Xg for 20 min, 4o) Bacteriocin activity was quantitated by spotting 20 ml of twofold serial dlilution of the culture supernatant that was adjusted to pH 6.5 and spotted onto the surface of MRS agar. The spotted agar was then over layed with 0.8 % MRS agar icoculated with Lactic acid bacteria . The plates were incubated at 37c for 24 h. The bacteriocin activity was determined by clear inhibition zone on the MRS agar.

Purification of Bactreriocin (Zamfir et al 1999)

Culture supernatant was obtained by centrifugation (8,000 Xg for 30 min , 4c) of L.acidophilus inculated MRS broth and incubated at 37c for 24h. Ammonium sulfate was added to reach 50% (wt/vol) and allowed to stir over night at 4c. The ammonium sulfate precipitate and bacteriocin collected by centrifugation at 10.000 Xg for 20 min and resuspended with ethanesulfonic acid .

Sensitivity to heat, pH and enzymes (Muriana, 1991)

Sample of bacteriocin was used for exposed to heat treatment of 65c for 40 min, 95c for 20 min, and 121c for 20 min and then were tested for re-

maining antimicrobial activity. Preparation were adjusted to various PH values in thr rang of 3 to 10.

The pH adjusted bacteriocin samples were incubated at 37c for 20 min and then neutralized to pH 6 and tested for bactriocin activity.

Susceptiblity of baceriocin to proteases was performed by incubating the bactriocin in the presence of proteinase(1 mg/ ml) at 37c for 1h. After incubation, the enzymes were inactivated by heat treatment at 65c for 30min and tested for bactriocin activity.

Acid and bile tolerance (Bogovic et al 1998) :

The strain incubated at 37c for 13h and then centrfuged (4.000 xg for 10 min, 4c).

The collected cell resuspended in sterile salin (0.85% Nacl).

The cell inoculated at 106 c.f.u/ ml and incubated at 37c for 1,2,3 h. respectively , the bacterial count were determined with MRS plates media.

Bile tolerance : was determined in MRS broth containing 0.3% bile acids, before testing for bile tolerance. strain were incubated at 37c for 18h in MRS broth without bile.

After centrifugated (4000 xg for 10 min, 4c), the collected cells were resuspended in sterile saline (0.85% NACL) and then inoculated into MRS broth containing 0.3% bile acids .Cultures were inculated at 37c. the bacteria were plated and enumerated after 24 h and 48 h of incubation.

SDS - polyacrylamid gel (schagger et al 1987)

The concentrated sample was electrophoresed on gel was washed three times in 250ml of H2O for 30 min and the gel was placed onto MRS agar medium.

Mice Immunization (Gill et al 2001)

Groups of BALB/ C mice weighting 20 - 30gm (each group contain 15 mice). Mice were fed milk based diets containing Bacterocin (1.5 X 10^8 c.f.u) isolated from gastric juice of healthy calves. The other group of mice was kept as control and only fed on milk based diets.

Serum ELISA (Shu and Gill , 2002)

In microtiter plates were coated with 100 ml of Bacteriocin antigen (5 µg/ml) and left over night at 4c.After washing with PBS, the 1:50 diluted serum samples were added and incubated at 37c for 1 h and washed three times with PBS , then 100 µof 1:100 anti mice horse radish proxidese conjugate was added and left to react at 37c for 30 min. The plates were washed again three times and finally 100µ of the substrate added.

After the colour developed 25µ of H_2So_4 were added to stop the reaction and plates were read at 490 nm.

RESULTS

In table 1 Bactriocin produced by *L.acidophilus* displayed antimicrobial activity against some of the tested strains. The bacteriocin was capable of inhibting most Gram positive pathogenic bacteria and Gram negative bacteria.

In table 2 the effect of enzyme , PH and Heat on the Bacteriocin. From data in table indicate that the inhibitory activity of bactriocin un affected by heating and 50% of activity still remained after a heat treatment of 121c for 20 min also indicate the bactriocin was to be sensitive to proteinase enzyme.

L.acidphilus bactriocin produced was completely stable at PH 6 and 7. 50% of activity remained after subjection to the various ph values between 3 - 9. As shown in table bacteriocin has exhibited excellent bile torlerance and enhanced growth in media containing ٢.. % bile

In table 3 clearified morbidity and mortality rates of mice post challenge.

Three mice were died from group 4, and two mice was died from group five, while one mouse was died from group seven and two mice was died from group eight.

The bactriocin antigen analysis by electrophesis (SDS - PAGE) was displayed in figure (1). Resulted in a single band with the molecular mass of 5.4 KDa.

Serum IgG ELISA results revealed that the cut of value was 0.71 (0.16 ± 0.54) when *E.coli* challenged , 0.70 (0.16 ± 0.53) when *S.typhimurium, K. Pneumoniae* and *B.Cereus* challenged.

In challenged mice with *Y.entericolitica, PS.aeruginosa, S.aureus,* and strept faecalis the cut of level of absorbance were 0.32 (0.4 ± 0.29), 0.25 (0.2 ± 0.22), 0.51 (0.9 ± 0.43) and 0.32 (0.4 ± 0.29) respectivily.

Table (1): Antimicrobial spectrum of bacteriocin from lactobacillus acidphilus against Gram positive and Gram negative bacteria .

Bacteria	Inhibition	Refernce
Gram negative		
E .coli 0:55	+	Lab isolate
K. pneumoniae	+	Lab isolate
S. typhimurium	+	R .strain
Y. enterocolitica	+	Lab isolate
P. aeruginosa	+	Lab isolate
Gram positive		
-B. cereus	+	R .strain
-S. aureus	+	Lab isolate
-St. Faecalis	+	Lab isolate

R.strain = reference strain

Table (2): Characteristics of bacteriocin

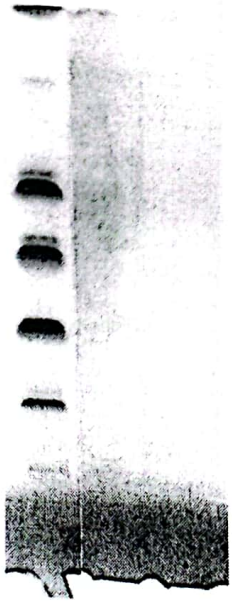
Trentment	Activity %
%Heat treat ment	100
65°c 140min	100
95°c 120min	50
121°c 120min	
PH	50
3	50
4	50
5	100
6	100
7	50
8	50
9	
Bile teorefaace	+ve (excellent exhibited)
Acid torlance	+ve (survived)
ProteinaseEnzyme	100 % sensitive

Table (3): Survival rate of mice protect with bacteriocin when challenged with different strains of Gram positive and Gram negative bacteria

Group No.	Type of organism	Treated bacteriocin	No. of dead mice	No. of protect mice	% of inhibition
1	*E .coli* 0:55	+	0	15	100
2	*K. pneumoniae*	+	0	15	100
3	*S. typhimurium*	+	0	15	100
4	*Y. enterocolitica*	+	2	12	80
5	*P. aeruginosa*+	+	3	13	87
6	*B. cereus*	+	0	15	100
7	*S. aureus*	+	1	14	93
8	*St. Faecalis*+	+	2	13	87

Table (4): Estimation of anti body titre in the serum of mice by ELISA test

Group No.	Negative serum	Type of infection	Mean of (cat off value)
1	0.066	*E .coli* 0:55	0.71 (0.16±0.54)100%
2	0.073	*K. pneumoniae*	0.70 (0.16±0.53)100
3	0.016	*S. typhimurium*	0.70(0.16±0.53)100%
4	0.070	*Y. enterocolitica*	0.32(0.4±029)87%
5	0.059	*P. aeruginosa*+	0.25(0.2±0.22)80%
6	0.085	*B. cereus*	0.70(0.16±0.53)100%
7	0.054	*S. aureus*	0.51(0.9±0.43)93%
8	0.062	*St. Faecalis*+	0.32 (0.4±029)87%



Tricine - SDS- PAGE : Gel stained with coomassia blue stain .

Lane (1) : Low molecular mass protein standard .

Lane (2) : Purified bacteriocin at 5.4 KDa .

DISCUSSION

BACTERIOCIN produced by *Lactobacillus* acidophilus has been partially characterized.

Bacteriocin is an important biodefense factor in the preventing, colonization and subsequent proliferation of pathogenic bacteria (Leer et al., 1995). (Zamfir et al., 2000 , Devust and Nevsens 2004) .

Bacteriocins are peptides or proteins which exhibit inhibitory activity against sensitive strains of bacteria, and act as antimicrobial peptide have been found to be widely distributed in microorganisms (Jack et al., 1995). (Foulquie et al., 2001, Marekova et a., l 2004) .

From table (2) Bacteriocin: produced sensitive to proteases and exhibited more acid and it`s evident that bile resistance, the acid tolerance of Bacteriocin is dependent upen the pH profile and the composition of the cytoplasmic membrane , which is largely in fluenced by the type of bacteria, type of growth media and the incubation condition . Bacteriocin have been shown to remain active at PH 3.0 or less . Bile to lerance is one of the most essential criteria for astrain to be used as probiotic culture, Bile acids have been shown to inhibit microorganisme (Zamfir et al., 1999). And (jack et al., 1995)

Survival in the host body, adherence and colonization are considered important properties for probiotic strains, so the ability of probiotics to establish themselves in the intestinal tract should enhance their ability to eliminate competitors using bacteriocin. (Leroy , F. et al., 2001 , Fuller, R. 1989) .

The bacteriocin display antimicrobial effect on bacteria such as *E.col.. S.typhimurium, B.cereus* spore forming bacteria (Gill et al., 20001).

Bacteriocin is also stable over wide range of PH. The heat and PH stability may be useful if the bacteriocin is to be used as an antimicrobial agant

The molecular mass of bacteriocin was estimated at 5.4 KDa, where as (Schagger et al., 1987) (Barafoot, 1984), Similarly (Leer, 1995) the molecular mass of bacteriocin migrated with molecular mass of 3.8 -6.5 KDa on SDS-PAGE

To characterize the function of the bacteriocin produced by *L.acidophilus*, it can be investigated by immunization and challenge experiments on mice, Bacteriocin protected mice showed 100% survivel percentage in *E.coli* and *S.typhimurium* and *K.preumonia* challenged, while 93% , 80% , 87% protection in *S.aureus, Y.entericolitica*, and strept fecalis challenged, wheres the unprotected mice showed survival rates ranged between 40% to 50% when challenged with different strains of gram positive and gram negative bacteria.

From result in table 3 elucidated the protective effect of bactriocin this result supported by (Strus et al., 2001) (and Sullivan et al., 2003) who suggested that this reduction may be associated with enhanced humoral and cellular immune responses.

The antibody titre against the gram positive and gram negative bacteria in the serum of mice proctect by bacteriocin and challenged by pathogenic microorganism as in table (4). were tested with IgG ELISA . The cut off point was 0.7 (0.16 ± 0.54) 100% protection in challenged the mice with *E.coli, S.typhimurum* and *K.pneumoniae* the cut off point was 0.5 (0.9±0.43) protection in challenged mice with *S.aureus* , but in challenged mice with *Y.entericolitica, PS.aeruginosa,* strept fecalis the cut off point were 0.51 (0.9±0.43) 0.32 (0.4±0.29) and 0.25 (0.2+0.22).

These results confirmed the sureriority of serum IgG ELISA, which also supported previous investigation which recommended serum IgG ELISA in term of convenience and sensitivity (Gill et al., 2001, Agarwal et al., 2003). In conclusion bacteriocin of lactobacillus acidophilus meets several of the criteria for use as a probiotic culture , which includes acid and bile tolerance . as will as the production of antagonistic substances. These characteristics may be advantageous for probiotic culture to be successful in colonizing and to compete with pathogens. (Nevsens and Vuyst 2004)

This study affirms that Bacteriocin of lactobacillus acidophilus display immuno enhancing properties in micro organism, challenged mice and demonstrates that oral delivery of this probiotic can promte in creased protection against a highly virulent enteric bacteria pathogen.

REFERENCES

Agarwal,R . Sharama N. Chaudhry, R . and Panigrahi Pp. (2003): Effect of oral baterocin of lactobacillus on Entnic micro flora. J. pediatr Gastroenterol Nutr. 36(3)397-402.

Barefoot , S..F. (1984): Purification and characterization of Lactobacillus acidophilus bactariocin. Antimicrob. Agent Chem. Other 26 : 328 - 334 .

Bogovic, B , Rogel: I , Nes I.f and Holo, H. (1998): Isolation and charac terisation of bacteniocin of lacto bacillus acidephilus Applied micro bilogy and Biotechvology 49:606-612.

De Vuyst, L. & Neysens, P. (2004): Biodiversity of sourdough lactic acid bacteria. Trends Food Scie Technol. In press.

Fuller, R.J. (1989): probiotics in man and animals .j. Appl. Bacterviol. 66: 365-378 .

Foulquié Moreno, M.R., Callewaert, R. & De Vuyst, L. (2001): Isolation of bacteriocins through expended bed adsorption using a hydrophobic interaction medium. Bioseparation, 10, 45-50.

Gill , H . Shu ,Q . and Cross, N. (2001): Protection against translocating salmonulla typhimurium infection in mice by feeding the immuno enhan ciny probiotic la ctobacillus med microbial Immuno : 190 (3) 97-104.

Gilliland S.E (1986) : Bacterial starter cultures for food . Boca Raton , Florida CRC' Press .

Hudaults , Lievins, Camard M.F and Serving, Al . (1997): Antagonistic ativity exerted in vitro and in vivo by lactobaally against Salmonella typhimurium infeetion. Applied Environ.l Microbio. 63,513-518.

Jack ,R. Tagg,J. and Ray, B.(1995): Bactevocins of gram positive bactona Microbiol rev. 59 : 171-200.

Kalchayanand N, Hanlin, M. and Ray ,B .(1992): Sublethal in Jury makes Gram negative and res is tant gram postivie bacteria Sensititve to the bacteriocins letter in applied microbial 15-239243 .

Klaenhammer ,T.R (1993): Genetics of bacteriocin produced by lactis a cid bacteria Fems. Microbial . Rev 12:39-86.

Leer, R.J. Vander, M, Van Giezen ,J.M and Noort (1995): Genetic analysis of noval bacteriocin produced by Lactobacillus acidophilus. Microbioloyy 141 : 1629 - 1635

Leroy, F. & De Vuyst, L. (2001): Growth of the bacteriocin-producing Lactobacillus sakei CTC 494 strain in MRS broth is strongly reduced due to nutrient depletion: introduction of a nutrients depletion model for the growth of lactic acid bacteria. Appl. Environ. Microbio. 67, 4407-4414.

Genetic analysis of noval bacteriocin produced by Lactobacillus acidophilus. Microbioloyy 141 : 1629- 1635 .

Marekov·, M., Laukov·, A., De Vuyst, L., Skaugen, M. & Nes, I.F. (2003): Partial characterization of bacteriocins produced by environmental strain Enterococcus faecium EK13. J.Appl. Microbiol. 94, 523-530.

Muriana P.M (1991): purification and partial charactenizatia of bacteriocin produced by lactobacilli acidophilus. Applied Envivon. Mitrobio. 57 , 114-121.

Neysens, P. & De Vuyst, L. (2004): Kinetics and modelling of sourdough lactic acid bacteria. Trends in Food Science and Technology. In press.

Schagger , H , and Von Jagow (1987): Tricine - Soclium dodecyl sulfate poly acry la mide ged electrophoresis in the range from I to 1oo Bichem . 166:368- 379 .

Shu Q, Gill H (2002): Immune protecton mediated by the probiotic lactobacillus agamst *Escherichia coli* 0157 : 47 infection in mice Fems Immunol Med Microbial 6:34 (1):59-64.

Strus , M . Pakosz, K. Prozondo, A. and Rozyn, K. E. (2001): Antagonistic activity of lactobaallus bacteria against anaerobic pathgens ned Dosw Mikrobiol : 53 (2)133-42

Sullivan,A. Barkholt ,Land Nord CE (2003): Lacto baullus acidophilus prevent anti biotic associabed ecological disturbances of Bacteroides fragilis in the intestine J. Antimicrob Chemother :52 (2)308-11.

Tahara, T. Kanatani, K. Yoshida, H . and Oshimura, M. (1992): Purification and som properties of bacteriocin produced by leactobacillus a cidophilus , biosci , biochem . 56 : 1212-1245.

Toba, T. Yoshioka, E. and Toht anew (1991): Heat latile bacteryiocin produced by lactsbacillus acidophilus . letters in Applied micro biology 12,106-108.

Zamfir, M,.Callewaert . Covnea, L. and Vatafu , I. (1999): Purification and cherac terizatio of bacteriocin produced by lactobaallus bacteriocin produced by lactobaallus acidophilus . j. of applied Micro biology 87, 923-931 .

Zamfir, M., Callewaert, R., Calina Cornea, P., Savu, L., Vatafu, I. & De Vuyst, L. (2000): Production kinetics of a bacterioncin produced by Lactobacillus acidophilus IBB 801. FEMS Microbiology Letters,190, 305-308.