PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

Pakistan Journal of Nutrition 7 (5): 689-694, 2008
ISSN 1680-5194

Characterization of *Lactococcus* Phages from Dahi Whey

Kashif Hussain[1], Tariq Masud[1], S. Maqsud[2] and Talat Mahmood[1]
[1]University of Arid Agriculture, Rawalpindi, Pakistan
[2]Quaid-I-Azam University, Islamabad, Pakistan

Abstract: For the production of yogurt and related products, the local and the commercial yogurt manufacturers of Pakistan are still, by and large using an undefined, unclassified mixture of lactic acid bacteria together with a large number of contaminated strains. Phages isolated in our part of world show different characteristics in terms of phage morphology, lytic patterns and burst size etc, compared to phages isolated elsewhere in the world cause serious problems to fermentation process and ultimate economic losses to dairy industry. In order to develop indigenous resistant starter cultures phages were isolated from samples of Dahi whey isolated phages were characterized on the basis of host range, latent period and burst size. Five virulent phages KT-1, KT-2, KT-3, KT-4 and KT-5 were isolated from twelve samples of whey using *Lactococcus lactis* strains as host. These phages caused lysis and ultimately retarded acid production of host strains. Three phages i.e. KT-1, KT-2 and KT-3 have shown multiple host specificity. Phages KT-1 and KT-2 have same latent period of 15 minutes but exhibited a slightly different rise period that is 20 minutes and 25 minutes respectively, the burst size of 90 and 65 phages per cell were detected for KT-1 and KT-2 respectively. KT-4 showed burst size of 80 phages per infected cell, whereas KT-5 showed burst size of 90 phages per cell. The results reveal that bacteriophages from this region are different from phages isolated in other parts of world.

Key words: *Lactococcus*, bacteriophages, Whey, yogurt

Introduction

Lactococcus bacteria have found their use in the manufacture of several fermented dairy products in which they are responsible for the acidity and characteristic flavor. Sufficient lactic acid formation by lactic acid bacteria during the manufacture of fermented dairy products is of utmost importance. Factors contributing to a slow starter are composition of milk, contaminating microorganisms, changes in fermentation behavior and most commonly encountered bacteriophages attack (Klaenhammer, 1984). The first to attribute the cause of starter failure to bacterial cell dissolving activities of bacteriophages. Since then failure due to phages have been observed throughout the world (Whithead and Cox, 1985).

Elaborate studies on various aspects of phage including morphological and structural characterization, host phage interaction, effect on lactic fermentation and product quality and genetic studies have been carried out in order to combat phage attacks in dairy industry (Klaenhammer, 1984). However, such a detailed study on the phages isolated from our indigenous dairy products such as Dahi, Lassi and Whey, has not been attempted despite its tremendous significance. The present work is concerned with the isolation and characterization of *Lactococcus* phages specifically from Dahi whey as it provides an excellent medium for the propagation of bacteriophages. The prime objective of this work was to isolate and characterize *Lactococcus* bacteriophages. The with respect to their of host range, latent period and burst size and to study the effect of these bacteriophages on lysis and ultimate reduction in acid formation.

Materials and Methods

Bacterial strains and media: Bacterial strains used in this study were isolated and characterized in the Department of Food Technology, University of Arid Agriculture Rawalpindi, Pakistan. The M-17 media was used in this study for the growth and maintenance of bacterial strains and M-17 broth was also used for the phage propagation. Phage suspensions that were used regularly in this research were kept refrigerated at 4°C in M-17 broth.

Whey samples: Twelve samples of whey were collected from different localities of Rawalpindi city. Samples were collected randomly in sterilized bottles. Samples were cooled on ice immediately after collection for transportation and brought to the laboratory for further analysis.

Isolation of bacteriophages: Isolation of bacteriophages was performed according to methods described by Jarvis (1989). First whey was centrifuged at 3000g for 20 minutes and supernatant was collected and filtered through 0.45 μm sterilized filter and filtrate was kept at 4°C for further isolation of bacteriophages. Aliquots (0.1

mL) of an overnight broth culture of bacterial host *Lactococcus lactis* were placed in sterilized test tubes. One drop (0.05 mL) of sterilized one molar calcium chloride (1M $CaCl_2$) was added to each tube followed by the addition of 0.1 mL of test filtrate.

After incubation at 37°C for 15 minutes, then top soft agar M-17 (2.5 mL per tube) was added into the tube. Tube contents were mixed by swirling gently and then immediately poured on the surface of set bottom (M-17) agar plates. Plates were incubated at 37°C and observed periodically for the appearance of plaques from three hours onwards.

Two well isolated plaques were separated with the help of sterile toothpick into a 0.5 mL broth M-17 chloroform was added 0.2 percent (V/V). After overtaxing for 60 seconds, the tubes were kept at 4°C overnight.

Large scale phage production: Phage stocks were prepared by addition of 0.5 mL of phage lysate and 0.1 mL of 1M $CaCl_2$ to 100 mL broth cultures in M-17 (Absorbance 0.5 at 650 nm) at 37°C, containing 1 percent host bacterium. During incubation at 37°C, lysis occurred after two hours and completed within three to four hours which was indicated by the optical density which reaches at 0.16 then lysate was centrifuged at 3000g for 20 minutes. Supernatant was collected and chloroform was added at the rate of 0.2 percent (v/v). After vortexing, the tubes were kept at 4°C for further examinations.

Growth and culture maintenance methods: The purity of cultures was tested according to the method proposed by Harrigan and McCance (1976). Cultures were inoculated in tubes with 10 mL of reconstituted sterile skimmed milk, using 15% inoculum and then frozen at 0°C before incubation for cell growth. Incubating at 30°C until milk coagulation made reactivation.

Test for bacteriophage presence: This test was carried out according to method of Terzaghi and Sandine (1975). 0.1 mL of cultures was placed in test tube containing 2.5 mL of M-17 broth. 0.5 mL of 1 M $CaCl_2$ was added to the test tubes. After slight agitation, the material was poured into Petri dishes with a solidified layer of M-17 agar. After solidification of the second layer, a drop of ultra purified whey was placed on its surface to test the presence of bacteriophages. Plates were then incubated at 30°C for 18 hours. Plaques of bacteriophages were observed where they caused the lysis of bacterial strains.

Isolation and storage of bacteriophages: Storage of bacteriophages was carried out according to the method of Terzaghi and Sandine (1975). Plaques were carefully removed from plates and transferred to the test tubes containing 0.1 mL of host culture and 0.05 mL of 1 M $CaCl_2$. Test tubes were then incubated at 30°C for six hours when cell lysis was observed. The lysis was indicated by the relative clearness of broth when compared with the controls. The controls were more turbid due to the presence of bacterial growth. Those tubes where lysis was observed were filtered through 0.45 µm membrane filter and stored at temperatures between 2°C and 5°C.

Loss in acid production: This test was carried out in reconstituted skimmed milk as described by Anderson and Meanwell (1942). For each bacterial strain under study, two controls and five test tubes were prepared with 10 mL of milk each. In control, 2% cultures were added. (Cultures were first incubated for 18 hours in reconstituted milk). Test tubes received 2% of cultures and 1 mL of filtered whey. All tubes were incubated in water bath at 30°C for two hours and then at 35°C for four hours. After that, acidity was determined by titration with 0.1 N NaOH using phenolphthalein indicator.

Addition of bacteriophages to commercial starters: This test was conducted by using the reconstituted skimmed milk at 11%. In this case each bacterial strain had one control and two test tubes for each fermentation hour. Test tubes received 1% of reactivated cultures and 0.5% of a mixture of all bacteriophages isolated in the samples. The controls were containing only starter cultures. All tubes were incubated at 30°C for 8 hours and a sample was taken every hour and acidity was determined by titration with 0.1N NaOH.

Isolation and sensitivity of cultures isolated from commercial starters: For this test reactivated commercial starters were used. Starters were successively diluted up to 10^{-9} in 0.1% peptone water.1 mL of each solution was plated in APT agar (APT broth agar). After overlaying with same medium, petri dishes were incubated at 30°C for 48 hours. Then isolated colonies were inoculated in milk until coagulation of milk. After coagulation, cultures were transferred to M-17 broth and incubated at 30°C for 18 hours. Cultures were then tested with all those bacteriophages, which were isolated in this study. All the cultures were tested using method of Hull (1977) to evaluate the host sensibility of bacteriophages.

Host range determination: Host ranges of isolated bacteriophages were determined by spot test with cross-reactions on all *lactococcal* host strains as described by Moineau *et al.* (1994). In this test, 2.5 mL of M-17 agar was mixed with 0.1 mL of actively growing *lactococcal* culture. After gentle mixing, the mixture was

Table 1: Bacteriophage presence in whey samples against *Lactococcus strains*

Whey Samples	*Lactococcus lactis* Starins				
	C1	C2	C3	P1	P2
A	-	-	-	-	-
B	+	+	-	-	-
C	+	+	-	-	+
D	-	-	-	-	-
E	-	+	-	+	-
F	-	-	-	-	-
G	-	-	-	+	-
H	-	-	-	-	-
I	-	-	-	-	+
J	-	-	-	-	-
K	-	-	-	-	-
L	-	-	-	-	-

+Lysis observed, _No lysis observed

poured onto the M-17 agar plate. After solidification, 20 µL of phage lysates were spotted on the lawns. Plates were incubated upright at 30°C for 24 hours and then examined for lysis.

Burst size and latent period: The percentage of adsorption, minimum latent period and average burst size was determined by the method of Loof *et al*. (1983).

Percentage of adsorption: Percentage of adsorption of phages to bacterial host was determined by using the method of Loof *et al*. (1983). The adsorption was allowed for 10 minutes and supernatants were filtered through 0.2 µm sterilized filters to separate unadsorbed phages and bacteria. Experiments were replicated thrice and mean adsorption values were determined from all the trials.

Burst size and latent period determination: Burst size and latent period were determined through following test. All host strains were grown to an optical density of 1.2 (600nm). Phages were added to hosts at multiplicities of infection ranging from 0.002 to 0.04. After the adsorption period, cells were centrifuged, resuspended in M-17 and diluted to 10^2 to 10^3 infected bacteria per mL. Suspensions were incubated at 30°C. Samples taken over time of 0 to 75 minutes were immediately titrated for phage determinations.

One step growth curves were constructed to determine the latent periods and burst sizes. Burst sizes were determined by dividing the mean plaque count of sample following the rise period by the mean plaque count of samples from the latent period. Mean values were determined by pooling means from three times replicated experiments for each phage.

Results and Discussion

This study was designed to isolate and characterize those bacteriophages, which are present in our environment and are causing serious damage to starter cultures and ultimate losses to dairy industry. Dahi whey provides very conducive medium for the *Lactococcus* bacteriophages. Twelve samples of whey were collected from different localities of Rawalpindi to check out the incidence of *Lactococcus* bacteriophages. These samples were then subjected to further analysis. First of all these samples were tested for presence of bacteriophages against *Lactococcus* strains.

Test for bacteriophage presence: Five strains of *Lactococcus lactis* were used in this study. Twelve samples were tested against all these strains to check the incidence of Lactococcus bacteriophages in those whey samples. Whey samples were centrifuged at 3000g for 20 minutes and then filtered through 0.45 µm sterilized filters. These samples were then assessed for the presence of bacteriophages. All twelve samples were spotted neatly on the lawns of five strains of *Lactococcus lactis*. It was observed that only five, out of twelve tested samples, showed presence of bacteriophages. The bacteriophages were found present in five samples of whey. These samples were B, C, E, G and I and phages from these samples were designated as Phages KT-1, KT-2, KT-3, KT-4 and KT-5, respectively in Table 1.

A variable size of plaque formation occurred. Large size plaques were selected for further characterization of phages. It was further observed from the table that the lytic activity of these bacteriophages was different against different strains. Phage KT-1 showed lytic activity against two strains of *Lactococcus lactis* and caused complete lysis. Whereas phage KT-2 caused lysis of three strains i.e.C1, C_2 and P_2, respectively.

On the other hand, phage KT-3 showed lytic activity against two strains. These strains were C2 and P1. Strain P1 was also the only strain that was lysed by the Phage KT-4. Phage KT-5 was able to attack only one strain that is P2.No bacteriophage was able to cause lysis of strain C3 (Tale 1). The possible reason for this lytic pattern may be difference of receptive sides on host, cell wall composition as well as the concentration of Ca++ ions that facilitates the adsorption of phage to host cell.

Loss in acid production: The addition of bacteriophages in any culture causes the lysis of culture which ultimately leads to loss in acidification ability of lactic acid bacteria. The normal acidification rate of milk is retarded in presence of bacteriophages. Loss of acid production of *Lactococcus* cultures was determined by using skimmed milk that was reconstituted at 11%. Selected five strains of *Lactococcus lactis*were used to determine the presence of bacteriophages in whey samples. For each tested strain, five test tubes were prepared to check their acidification ability in presence of

Table 2: Comparison of rate of acid production among control and phage containing samples incubated at 35°C for 6 hours

	Treatments					
Strains	Control	KT1	KT2	KT3	KT4	KT5
C1	0.573LM	0.31OP	0.29OPQ	0.59L	0.63JKL	0.61KL
C2	0.82I	0.25PQ	0.32OP	0.42N	0.67JK	0.69J
C3	1.41CDE	1.15CDE	0.98G	1.07EF	0.90H	1.11DE
PI	1.43A	1.2BC	1.093EF	0.51M	0.42DE	1.12DE
P2	1.26B	1.03FG	0.22Q	0.98G	1.19BCD	0.36NO

*Means having same letters are statistically non significant at 5% alpha, *DMR test at LSD value 0.07304 and SD 0.02582, *Values are mean of three replications

Table 3: Addition of Bacteriophages to commercial starter cultures

Commercial starter	Treatments							
cultures	1 hr	2hr	3 hr	4hr	5hr	6hr	7hr	8hr
BD1	0.42^	0.55YZ[\]	0.63VWXY	0.74TUV	0.92OPQR	1.08JKLM	1.19GHIJK	1.31DEFG
BD2	0.46\]^	0.6WXYZ[	1.033LMNO	0.82QRST	1.04LMN	1.12IJKL	1.23FGHI	1.41BCDE
BD3	0.51YZ[\]^	0.59TUVW	0.71PQRS	0.900PQTST	1.10ILKL	1.93GHIJ	1.307DEFG	1.517ABC
BD4	0.43]^	0.54YZ[\]^	0.63VWXY	0.93NOPQ	1.09KLM	1.21GHI	1.4CDE	1.52AB
O1	0.45]^	0.49[\]^	0.59WXYZ	0.98MNOP	1.18HIJK	1.31DEFG	1.42BCD	1.59A
O2	0.52YZ[\]^	0.62VWXYZ	0.71TUVW	1.02LMNO	1.21GHI	1.41BCDE	1.503ABC	1.6A
O3	0.51YZ[\]^	0.63VWXY	0.79STU	0.63VYXY	0.50Z[\]^	0.50]^	0.49[\]^	0.49[\]^
O4	0.49[\]^	0.58XYZ[\	0.76TU	0.78TU	1.02LMNO	1.123IJKL	1.2GHI	1.30DEFGH
O5	0.54YZ[\]^	0.69UVWX	0.81RST	0.95NOP	1.2GHIJ	1.29EFGH	1.33DEF	1.42BCD
D1	0.60WXYZ	0.70YUVWX	0.82QRST	0.71TUVW	0.55YZ[\]	0.50Z[\]^	0.48[\]^	0.44]^
B	0.51YZ[\]^	0.61WXYZ	0.81RSTU	0.97MNOP	1.19GHIJK	1.21GHI	1.307DEF	1.420BCD

*Means having same letters are statistically non significant at 5% alpha, *DMR test at LSD value 0.1019 and Standard Deviation 0.365, *Values are mean of three replications

bacteriophages. The acidity was determined after a period of 6hrs of incubation at 35°C using 0.1N NaOH with the help of phenolphthalein indicator (Table 2).

These results further confirm the results obtained from test of loss in acid production, All those phages which were isolated from whey samples confirmed their presence by attacking the same strains and causing their lysis. These results indicate that rate of acid production in control tubes was steady. Whereas those strains which were susceptible to specific bacteriophages, their rate of acid production declined with the passage of time. It was observed that lytic activity started approximately after one hour of infection in the host strain which caused the decrease in acidity and after four hours culture was almost completely lysed. This may be attributed to the repression of host protein synthesis. These results show that in the presence of bacteriophages, there was a considerable reduction in the acidification rate. Reduction in the production of lactic acid above 10% indicates the presence of bacteriophages. These findings correlate with the findings of Anderson and Meanwell (1982).

Addition of bacteriophages to commercial starters: Once the bacteriophages were isolated from whey samples and their effect on *Lactococcus* strains was established, further studies were conducted to evaluate the effects of these bacteriophages on commercial starters. For this purpose ten samples of commercial starters were selected. Skimmed milk reconstituted at 11% was used in this test. In test tubes, 1% of commercial starter and 0.5% of mixture of all isolated bacteriophages was mixed. Acidity was determined using 0.1N NaOH with phenolphthalein indicator after an interval of one hour (Table 3).

These results show that most commercial starters were not sensitive to inoculated bacteriophages except O3 and D1 starters. According to our studies, results after 8 hours were qualitatively identical to those obtained after 6 hours. Quantitative difference was higher after 8 hours of incubation. It indicates that incubation for 8 hours generated more sensitive results than 6 hours. Although some authors have suggested that incubation for shorter periods (6hours) was more adequate to evaluate the starter behavior in presence of bacteriophages (Stadhouders and Leenders, 1984).

Mechanism of phage resistance that have been found in *Lactococci* include super infection immunity by prophage, reduction in adsorption efficiency, restriction-modification systems and abortive infection. Several of these mechanisms are plasmid encoded (Sanders, 1988). Investigations of the resistance mechanisms the starter strains are currently underway. Strains that demonstrate powerful mechanisms of phage resistance may become the prime candidates for use in other starter systems. Additionally, if these mechanisms are plasmid-linked, they may be useful for the genetic construction of new phage resistant strains (Daly and Fitzgerald, 1987).

Table 4: Sensitivity of strains isolated from starter culture BD1 against all isolated bacteriophages

Isolated Strains	KT1	KT2	KT3	KT4	KT5
C10	-	-	+	+	-
C11	-	-	+	+	-
P11	-	+	+	+	-
P21	-	-	+	-	+
P31	-	-	+	-	-
C20	-	+	+	+	-
C21	-	-	+	-	-

Table 5: Sensitivity of strains isolated from starter culture BD2 against all isolated Bacteriophages

Isolated Strains	KT1	KT2	KT3	KT4	KT5
C10	-	-	+	+	+
P21	-	-	+	-	-
P31	-	+	+	+	-
C20	-	-	-	+	+
T3	-	-	+	+	-

Table 6: Sensitivity of strains isolated from starter culture BD3 against all isolated Bacteriophages

Isolated Strains	KT1	KT2	KT3	KT4	KT5
M17-1	-	-	+	-	-
M17-2	-	+	+	-	-
P10	-	-	+	-	-
P11	-	-	+	+	+
C11	-	-	+	-	-
T3	-	-	+	+	-

Table 7: Sensitivity of strains isolated from starter culture O3 against all isolated Bacteriophages

Isolated Strains	KT1	KT2	KT3	KT4	KT5
M17-1	-	-	+	+	-
M17-4	-	-	+	+	-
C7	-	-	+	-	-
P31	-	+	+	-	-

Table 8: Sensitivity of strains isolated from starter culture O4 against all isolated Bacteriophages

Isolated Strains	KT1	KT2	KT3	KT4	KT5
P31	-	-	+	+	-

Table 9: Host specificity of Lactococcus bacteriophages

	Lactococcus Phages				
Starter Cultures	KT1	KT2	KT3	KT4	KT5
C1	+	+	-	-	-
C2	+	+	+	-	-
C3	-	-	-	-	-
P1	-	-	+	+	-
P2	-	+	-	-	+

Sensitivity of commercial starter isolated cultures: Since culture rotation is one of the methods used in industrial process to control bacteriophage attack so it is very important to determine the sensitivity level of starters bacteria to the bacteriophages found in industry. Keeping in view, the importance of this work, strains were isolated from commercial starters BD1, BD2, BD3, O3 and O4. There was a wide variation in sensitivity among isolated strains (Table 4). No starter isolate was sensitive to phage KT-1. On the other hand, almost every isolate (except for isolate 3 from starter BD2) was sensitive to phage KT-3. The absence of infective phages in certain isolates may be attributed to the non utilization of such cultures in industry. These findings correlate the findings of Hemming *et al.* (1968). Also if the tested starters were only recently used in the plant, there was no sufficient time for the multiplication of bacteriophages to be experimentally detected. The sensitivity of various strains isolated from starter cultures shown in tables given in following pages.

Host range studies: *Lactococcus* phages with multiple host range have been isolated by several workers (Jarvis and Meyer, 1986). Three out of five phages isolated in this study showed multiple host range (Table 9).

Prevalence of the phages capable of attacking more than one host is due to the commonly adopted practice of using multiple strain starters. Moreover, it is known that phages may adapt to the original non permissible host by a range mutation, which can alter its adsorption specificity or by host controlled modification.

Burst size and latent period: The infection cycle of bacteriophages were characterized by their one step growth kinetics. Phages KT-1 and KT-2 have same latent period of 15 minutes but exhibited a slightly different rise period that is 20 minutes and 25 minutes respectively. The burst size of 90 and 65 phages per cell were detected for KT-1 and KT-2 respectively (Fig. 1 and 2). On the other hand phages KT-3, KT-4 and KT-5 showed similar growth kinetics. The latent period for these three phages was calculated 25 minutes, their rise period was 30 minutes and however all these phages exhibited slightly different burst sizes. The burst size for phage KT-4 was calculated as 75 phages per cell.KT-4 showed burst size of 80 phages per infected cell. Whereas KT-5 exhibited burst size of 90 phages per cell (Fig. 3). The difference in latent period and burst size may be due to the fact that it is very difficult to obtain an exact correlation between colony forming units and number of cells in these microorganisms.

Composition of the medium strongly influences the growth kinetics of *Lactococcus* phages. A drastic decrease in the burst size was observed in complete medium in the presence of citrate (Cherry and Watson, 1994).

Shorter latent periods or more liberation on burst or both are characteristics of phage races that develop quickly and achieve high populations in cheese whey. These phages can cause complete failure of starter culture even when present in low concentration at the start of cheese making. The differences observed in burst sizes of the phages KT-3, KT-4 and KT-5 in spite of their identical latent period may be due to the disparity in the

individual eclipse phase of these phages. Composition of the medium strongly influences the growth kinetics of *Lactococcus* phages. The latent period, rise period and burst size of a *Lactococcus lactis* phage on a complete medium containing tryptone, yeast extract and glucose have been reported as 31 to 33 minutes, 15 to 18 minutes and 90 phage particles, respectively, whereas on a deficient medium that lacked tryptone, the latent period o phage growth increased 55 minutes, rise period decreased to 10 minutes and average burst size decreased to 24 phage particles. Also a drastic decrease in the burst size was observed in complete medium in the presence of sodium citrate (Cherry and Watson, 1994).

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