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Bacteriocin production by probiotic bacteria from curd and its field application to poultry

V. Veera Jothi*, K.T.K. Anandapandian and T. Shankar***

**Post Graduate Department of Microbiology, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, Tamilnadu, India*
***Agriculture Research Institute, Tanzania*

ABSTRACT

The present study was undertaken in the lactic acid bacilli isolated from curd samples and its probiotic efficiency was checked by feed supplement to poultry. A total number of 45 LAB (lactic acid bacilli) were isolated from the curd sample and the best bacteriocin producing Lactobacilli strain was selected by antibacterial activity assay, bile tolerance test, acid tolerant test and H_2O_2 content. Among the isolates the probiotic from raw curd (Lactobacillus sp VJ 32) was used for the dietary supplement to poultry. The isolate was evaluated for poultry feed supplement the result shows that the comparison with control are increased in weight and better feed supplement efficiency has been determined in chicks.

Keywords: Lactobacilli, LAB, Probiotics, Nutraceuticals, Feed, Poultry.

INTRODUCTION

Probiotic terms derived from Greek words Pro (favor) and bios (life). Probiotics are defined as live microbial feed supplements that improve the health of man by its valuable secondary products [1]. Lactic acid bacteria (LAB) are studied as one of the main probiotics. Probiotic organisms are *Lactobacillus, Lactococci, Bifidobacteria* and *Saccharomyces.* Probiotic bacteria release a variety of chemical compounds that are inhibitory to both gram-positive and gram-negative bacteria [2]. These include bacteriocins, sideropheres, lysozymes, proteases, hydrogen peroxides etc. Bacteriocins are proteinaceous compounds produced by a wide range of bacteria exhibiting antimicrobial activity against a select range of other bacteria [3].

The organisms must be able to survive the unfavorable environment of GIT, which benefits resistance to acid and bile [4]. *Lactobacillus* spp., have shown to reduce total coliform counts in wastewater facilities by 80% after 4 months of treatment [5].

The poultry Industry is now facing a ban for the use of antibiotic feed additives for disease prevention and growth enhancing supplements. Probiotics were used to overcome this problem. To maintain the intestinal microflora balance in animals it is important to prevent diseases by controlling the overgrowth of potentially pathogenic bacteria. The control of infections through anon antibiotic approach is urgently requested. The natural bacterial flora (Eg. probiotic bacteria) represents a promising alternative therapy. Probiotics were defined as "living microorganisms that upon ingestion in certain numbers exert health effects beyond inherent basic nutrition". Probiotics offer a promising alternative to chemicals and antibiotics in poultry. The probiotic treatment would be advisable as a harmless alternative to antibiotic, due to lack of risk of toxicity and anti bio-resistant spread [6].

Large numbers of lactobacilli may convert fermentable sugars quickly thus preventing this energy being used by pathogens. The intestinal growth of all other kinds of non intestinal prokaryotic and eukaryotic pathogens (such as yeasts and protozoa) is strongly inhibited by abundant probiotic fermentation in the small bowel [7]. Beneficial effects include control of diarrhea, alleviation of lactose intolerance [8], inhibition of intestinal pathogens [9]. The objective of this study was to isolate potential probiotic bacteria from curd and its application as poultry feed supplement.

MATERIALS AND METHODS

Collection of samples

The curd samples were collected from Sivakasi, in a sterile container.

Isolation of lactic acid bacteria (LAB)

The LAB colonies were isolated from curd by serial dilution and patch culture method in Mann Rogoso Sharpse **(**MRS) agar medium.

Patch culture

The Patch culture method was used for the isolation of LAB, from the isolated colonies using serially diluted plates. MRS Agar plates were prepared. Using sterile tooth picks the isolated colonies were gently touched and the colony was simply streaked by small line in the agar plates. In a single agar plate 15 colonies were patched. The plates were incubated at 37ºC for 48 hrs.

Identification of bacteriocin producing LAB

All LAB cultures isolated from MRS were submitted to an initial screening to verify the presence of antagonist activity using various inhibition methods.

Well diffusion method

The cultures of the indicator strains (*Staphylococcus aureus, Klebsiella, Pseudomonas, E. coli, Proteus* and *Bacillus*) were prepared by pouring 2ml of the inoculum onto MSA plates to completely cover the surface of the agar. Six mm diameter wells were punched into the agar using sterilized well cutter, which were cut to obtain a 6mm diameter bore. 20µl of each probiotic inoculum (10^3CFU/mL) was carefully pipetted into each well. The diameter of the inhibition zones around the wells were recorded in millimeters after incubating the plates for 48 h at 37°C [10, 11].

Spot-on-the-lawn technique

All LAB cultures isolated from MRS were submitted to an initial screening to verify the presence of antagonist activity using the spot-on-the-lawn technique. An aliquot of 2ml of each

LAB culture previously inoculated in MRS broth was spotted on plates containing 10ml of culture media and was incubated at 37ºC for 24 h. After incubation, the plates were overlaid with 8ml of BHI semi-solid agar (0.8g/100ml of bacteriologic agar) inoculated with 10^5 CFU/ml of a culture of indicator. The plates were then incubated at 37ºC for 24 h. The presence of a distinct inhibition zone around the spots was considered a positive antagonistic effect. The best antibacterial effect showing strains LAB VJ 15 and LAB VJ 32 were used for further studies.

Morphological, physiological and biochemical characteristics of test organisms (LAB VJ 15 and LAB VJ 32)

The morphological, physiological and Biochemical tests were performed for the identification of the test organism used in this study.

Effect of antimicrobial activity

The isolated strains (LAB VJ 15 and LAB VJ 32) were analyzed for Antimicrobial activity against *Staphylococcus aureus, Klebsiella, Pseudomonas, E. coli, Proteus* and *Bacillus* using well diffusion method. To check the antimicrobial activity, the MRS agar plates were overlaid with 7ml of soft MRS agar inoculated with 20ml of overnight active culture of indicator strains. Different wells were made in agar. Wells were filled with 20µl cell free broth of 24 h old cultures obtained by centrifuging the culture broth at 5000rpm for 15 min. The broth was neutralized to pH 6.5 and it was also inoculated into wells. The diameter of zone of inhibition extending laterally around the well was measured and a clear zone of 1mm or more was considered positive inhibition.

Curdling of milk

The Milk sample was pasteurized using sterile conical flask. After pasteurization, the Milk was distributed to sterilized tubes. The test culture was inoculated to the tubes and the tubes were incubated at room temperature for 24 hours.

Selection of potential probiotics

Acid tolerance

The acid tolerance of Lactobacilli was studied in different pH. The solutions were prepared by adjusting the hydrochloric acid (HCl) solution to pH levels of 2, 3 and 4 in double distilled water. Sterile double distilled water (pH 6.4) served as a control. Solutions were prepared in 100ml volume and sterilized at 121ºC for 20 min and stored at room temperature until used. After thorough mixing, 10ml of each pH solution was taken in sterilized test tubes. A cell suspension of selected *Lactobacillus* cultures containing about 10^{10}cells/ml was added to each pH solution of 2, 3 and 4 and control (pH 6.4) and mixed. One milliliter from each pH solution was taken after 1, 2, 4 h and serial dilutions were prepared using 0.85% sterile saline. Appropriate dilutions were pour plated in MRS agar and incubated aerobically at 37ºC for 72 h [4].

Bile tolerance

The bile salt solutions were prepared using Oxgall powder (HiMedia). The powder was rehydrated by preparing 10g dry powder base in 90ml distilled water (equivalent to rehydrated ox bile). From this solution, final concentrations of 1% (half) and 2% (maximum) were prepared. Sterile double distilled water without oxgall (pH 6.4) was used as control. All solutions were autoclaved and stored at room temperature until used. 10ml of each solution was transferred into sterile test tubes. Cell suspensions containing about 10^4cells/ml was added to each solution, i.e., 1%, 2% and control and incubated at 37ºC aerobically. One ml of culture was taken out from each tube after 3, 8 and 12 h time intervals and dilutions were prepared in sterile 9ml, 0.85%

saline blanks. Appropriate dilutions were pour plated in MRS agar and incubated aerobically at 37ºC for 48 h [4].

Tolerance to hydrogen peroxide

Strains were grown on MRS agar for 24 h. The overnight cultures were suspended at the level of 10^7 Cfu/ml in isotonic saline (sodium chloride) and incubated with 0.4mM hydrogen peroxide (30 wt. % solution in water at 37ºC. At 30 min time intervals, the removed aliquots were plated onto MRS plates and the number of viable cells was estimated by using the semi quantitative method. The incubation of MRS agar for the cultivation of Lactobacilli was performed at 37ºC for 48 h [12].

Tolerance to NaCl concentration

For the determination of NaCl tolerance of isolated Lactobacillus, 5 test tube containing MRS broth were adjusted with different concentrations (1, 2, 3, 4 and 5%) of NaCl. After sterilization each test tube was inoculated with 1% (v/v) fresh overnight culture of Lactobacillus and incubated at 37°C for 24 hrs. After 24hrs of incubation their growth were determined by observing their Optical Density [13].

Animal studies

Chicks

Five day old chicks were purchased from Poultry Company, at Thalavaipuram near Rajapalayam, Tamilnadu, India.

Experimental design

The chicks were divided in to two groups. One group was treated with test probiotics LAB VJ 32

Preparation of probiotics for field application to poultry

The selected probiotic bacteria (LAB VJ 32) were transferred in MRS broth. The probiotic bacterium was cultured aerobically at 37ºC for 48 h. The bacterial cells were collected by centrifugation (8000rpm for 10 mins), washed twice and then suspended in Phosphate Buffer Saline (PBS; 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.0). The total viable count of the washed bacterial cells suspension was used to probiotic treatment. The feed used in this experiment was a commercial formulated feed pellet obtained from the poultry industry and contained protein, energy, calcium, phosphorous, vitamins (A, B, D, E and K), liver powder, lycine, methionine, NaCl and toxin binder. Probiotic was mixed with the Feed. The LAB VJ 32 (*Lactobacillus* spp.) was used as a probiotic for the experiment, while the pellet was used without probiotics as the control.

Feeding

During the course of the experiment, the Chicks were fed with an excess ration twice a day (at 40:40) for each group.

Growth Evaluation

On every 7 days the chicks were weighted. Weight gain (WG), Feed Conversion Ratio (FCR), was determined using the following equation:

$$WG = 100 \times \frac{Wt \,.W_0}{Wo}$$

$$\text{Feed Consumption Ratio (FCR)} = \frac{\text{Feed consumption}}{\text{Weight gain}}$$

Where Wt is the final body weight; W_0 is initial bodyweight.

RESULTS

Isolation of lactic acid bacteria

Thirty five different colonies were isolated from curd sample using MRS agar medium, the colonies were creamy white, transparent and smooth round in shape (Fig. 1).

Patch culture

The antimicrobial activity of the selected strains was identified through Patch culture method (Fig. 2). The best antibacterial activity showing two strains (LAB VJ 15 and LAB VJ 32) were selected and they were confirmed as *Lactobacillus* based on its morphological (Table.1), physiological and biochemical characteristics (Table.2). The strain was gram positive, rod shape, non-motile, non-spore forming and facultative anaerobic lactic acid bacterium. The pure culture of test organism was sub cultured every 15 days throughout the studies (Fig. 3).

Effect of antimicrobial activity

The two *Lactobacillus* sp. (LAB VJ 15 and LAB VJ 32) was found to show an antibacterial activity in the well diffusion assay. The antibacterial substance produced by *Lactobacillus* inhibited pathogen such as *Staphylococcus aureus*, *Klebsiella* sp, *Pseudomonas* sp, *E. coli*, *Proteus* sp, *Bacillus* sp and *Salmonella* sp. The inhibitory effect was significant against *Gram* positive strain of *Bacillus*, the inhibitory effect was significant against of *Gram* Negative bacteria, *Salmonella* sp (12mm) (Table.3).

Acid tolerance

The survival of *Lactobacillus* strains at pH 2, 3 and 4 was observed under 1, 2 and 4 hrs of incubation. None of the strains survived at pH 2 for any time period (data not shown). LAB VJ 32 survived at pH 3 for 4 hrs (3.11 log cfu/ml) whereas no growth was recorded in LAB VJ 15. Both strains showed consistency in terms of tolerance to pH 4. Survival at pH 3 was promising for all strains. Survival at pH 4 is significant as ingestion with food or dairy products raises the pH in stomach to 3.0 or higher (Table.4).

Bile tolerance

The bile tolerance efficiency of LAB VJ15 and LAB VJ 32 sensitive at 1 and 2% bile concentration under 12 hr incubation period. Among the two isolated strains, LAB VJ 32 showed comparatively better tolerance at 1 and 2% concentrations (4.77 and 3.01 cfu/ml) respectively for 8 h (Table.5).

Tolerance to H_2O_2

It shows that the tolerance pattern of LAB VJ15 and LAB VJ32 on 0.4mM hydrogen peroxide showed that the tolerance condition was moderately by 4 hrs (1 and 3.01 cfu/ml respectively), but LAB VJ32 tolerated by 6 hrs (1 log cfu/ml) (Table.6).

Tolerance to NaCl concentration

Sodium Chloride is an important physicochemical factor for bacteriocin production. The NaCl effect on growth of LAB VJ15 and LAB VJ 32 in the medium with various NaCl concentrations

was studied (1 to 5%). Both the organisms (0.8150 and 0.926 OD value) were tolerated at 1% NaCl and lowered the growth (0.083 and 0.085 OD) was found at 5% NaCl (Table.7).

Probiotic efficiency to poultry

Table 8, 9 & 10 shows the Probiotic efficiency, Weight gain and Feed Conversion Ratio of Chicken. The values were calculated by using different equations. On 41 days of growth weight gain of chicken (nearly 100Gms) was increased when compared with control (Fig. 4).

DISCUSSION

Bacteriocins produced by probiotics are protein nature, ribosomally synthesized by gram positive bacteria. They have considerable attention as food preservatives and as potential replacement of antibiotics.

The antimicrobial activity of the selected strains was identified through patch culture method. The best antibacterial activity showing two strains (LAB VJ 15 and LAB VJ 32) were selected and they were confirmed as *Lactobacillus* based on its morphological, physiological and biochemical characteristics. The strain was gram positive, rod shape, non-motile, non-spore forming and facultative anaerobic lactic acid bacterium. Similar work was done by Kim *et al*. [14] where thirty-three samples of raw milk and eighteen samples of soft cheese made with raw milk were collected directly from dairy farms of the Vicosa region, Minas Gerais State, Brazil. All samples were ten-fold diluted on NaCl solution (0.85 g/100 ml) and pour plated on de Manne Rogosae Sharpe (MRS, Difco, and Loveton Circle Sparks, MD, USA).

The spot-on-the-lawn method is widely used to detect the proteinaceous nature of antimicrobial substances produced by LAB [15, 16, 17]. This method is advantageous in that proteinaceous substances can be identified even in cultures that produced small inhibition halos. In our study we isolated the strains of LAB by the spot-on-the-lawn method.

In our study antibacterial substance produced by *Lactobacillus* inhibited pathogen such as *Staphylococcus aureus, Klebsiella* sp, *Pseudomonas* sp, *E. coli, Proteus* sp, *Bacillus* sp and *Salmonella* sp. the inhibitory effect was significant against Gram positive strain of *Bacillus*, the inhibitory effect was significant against of Gram negative bacteria, *Salmonella* sp (12mm). *Lactobacillus plantarum* KCTC 3635 was also used as indicator bacteria for the identification of bacteriocin. Indicator plates were prepared by inoculation approximately 107 cells of indicator bacteria to 15 ml soft agar (0.8%, NA or MRS agar; Difco) containing 0.002% methylene blue [18].

The survival of *L. casei* strains at pH 1, 2 and 3 was observed for 0, 1, 2 and 3 h. while none of the strains survived at pH 1 for any time period, only strains NCDC 17, isolate C1 and Y could survive pH 2 for 1 h. All seven strains except for NCDC 17 showed consistency in terms of tolerance to pH 3. The residual counts were 10^7cfu/ml even after 3 h of incubation. Survival at pH 3 was promising for all strains but not at pH 2. Survival at pH 3 is significant as ingestion with food or dairy products raises the pH in stomach to 3.0 or higher [19].

Similar results were found in this study, but there was no growth was observed in 2 pH. Goldin *et al*. [20] also reported survival of *Lactobacillus* GG at pH 3, almost complete loss of viability for *L. casei* 212.3 and F19 strains and *Lactobacillus* GG at 3 h interval at pH 2.5. Jacobsen *et al*. (1999) reported that out of 44 *Lactobacillus*, none of strain could replicate at pH 2.5.

The bile tolerance efficiency of LAB VJ15 and LAB VJ 32 sensitive at 1% and 2% bile concentration under 12 hr incubation period. Among the two isolated strains, LAB VJ 32 showed comparatively better tolerance at 1% and 2% concentrations respectively for 8 h. The result of isolates high tolerance to bile in the study was different from other reports. The probable reason was that the isolates were from animal feces and intestines and had more chance to be exposed to bile salts. The inhibitory compound was found to be heat labile, losing activity when temperatures were raised above 37°C. The inhibitory activity was seen only in a narrow pH range, when adjusted to pH values lower than pH 4 or above pH 8 all inhibitory activity was lost. The relative heat and pH stability of the inhibitory compound lends support to the assertion that the inhibitory compound was a protein and as such was a bacteriocin. The NaCl effect on growth of LAB VJ15 and LAB VJ 32 in the medium with various NaCl concentrations was studied (1 to 5%). Both the organisms were tolerated at 1% NaCl and lowered the growth was found at 5 % NaCl in our study.

The inhibitory compound was found to be less salinity concentration, losing activity when NaCl concentration was increased above 5%. The inhibitory activity was seen only in a narrow NaCl volume, when added NaCl volume to lower than 0.5% NaCl or 2% NaCl all inhibitory activity was lost. The relative salt concentration lends support to the assertion that the inhibitory compound. The sodium chloride is an important physiochemical factor for any marine and esturine animal to maintain the osmoregulation [13].

The tolerance pattern of LAB VJ 15 and LAB VJ 32 on 0.4mM hydrogen peroxide showed that the tolerance condition was 4 hrs for LAB VJ 15, but LAB VJ32 tolerated for 6 hrs. The effect of potential production of hydrogen peroxide by *Lactobacillus* was discounted, since addition of catalase to the supernatant did not eliminate the inhibitory effect [12]. The most phenomenons were proportional to the concentration of hydrogen peroxide (*i.e.*, the higher concentration of hydrogen peroxide the lower the survival). Although the resistance of the antioxidative strains was not comparable with the resistance of *S. typhimurium* (the latter was resistant to 0.4mM hydrogen peroxide killing even after 48 h and resistant to 1.0mM hydrogen peroxide after 24 h, data not shown), the antioxidative strains have significantly increased resistance to harsh media compared with the non antioxidative strain.

The Probiotic efficiency and Feed Conversion Ratio of Chicken were calculated by using different equations. On 41 days of growth weight gain of chicken (nearly 100gms) was increased when compared with control. The results of body weight gains support the finding of Kabir *et al.* [21] who found that live weight gains were higher in probiotics fed group as compared to birds having no probiotics. In their study they found that the broiler chicks gained lowest body weight when fed with diet deficient in vitamin mineral premix.

The results revealed that probiotics, vitamins and minerals had positive effect on live weight gain in comparison to normal balanced ratio. Highest body weight gain was found in the birds of group D (vitamin fed group) compared to other groups. The use of probiotics in the diet has become a phenomenon for maintenance of normal growth and health of birds. Feed-type probiotic products have been demonstrated to help and maintain a positive balance of intestinal microflora resulting in the improvements in health and weight of the chickens throughout their short life span [22].

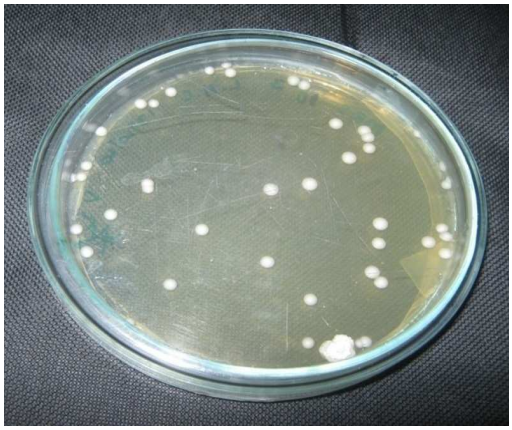

Fig. 1. Bacterial colonies isolated in MRS medium

Fig. 2. Patch culture of *Lactobacillus* (LAB VJ 15) showing Bacteriocin activity

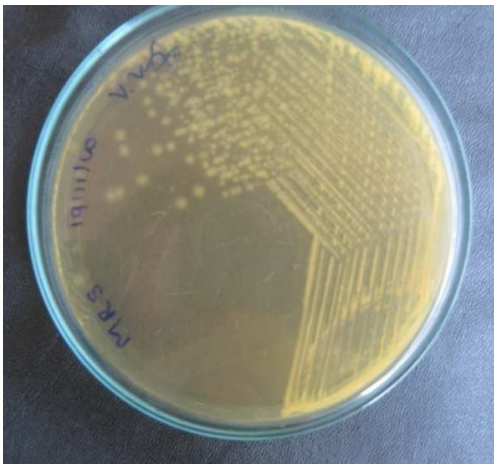

Fig. 3. Pure culture of LAB VJ 32

A – Initial Day; B – After 42; DaysC – Control; D - Test

Fig. 4. Probiotic efficiency to poultry

Table. 1. Morphological and physiological characters

Test	**Result**
Gram's staining	Positive, Rod
Motility	Non-motile
Morphological characteristics	Small, circular and smooth
Spore	Non-spore forming
Pigment	_
Bile solubility	Insoluble

Table. 2. Biochemical characteristics of test organisms (LAB VJ 15 & LAB VJ 32)

TEST	RESULT
Gram's staining	Positive, Rod
Motility	Non-motile
Indole	−
Methyl red	−
Voges Proskaur	−
Citrate utilization	+
Catalase	−
H_2S Production	−
Nitrate reduction	−

Table. 3. Antimicrobial activity of Isolates

PATHOGENS TESTED	ZONE OF INHIBITION (mm)	
	LAB VJ 15	LAB VJ 32
Staphylococcus sp	8	8
Salmonella sp	10	12
Klebsiella sp	8	7
Pseudomonas sp	9	11
Escherichia coli	7	9
Proteus sp	6	8
Bacillus sp	11	12

Table 4. Acid Tolerance of lactobacillus (log cfu/ml)

STRAINS	Time Intervals	pH		
		2	3	4
LAB VJ 15	1	-	6.02	7.78
	2	-	3.01	4.77
	4	-	-	3.01
LAB VJ 32	1	-	6.98	9.03
	2	-	4.77	6.98
	4	-	3.01	3.01

Table 5. Bile tolerance of *Lactobacillus* (log cfu/ml)

STRAINS	Time Intervals	Bile concentration	
		1%	2%
LAB VJ 15	3	6.02	4.77
	8	3.01	1
	12	-	-
LAB VJ 32	3	6.02	3.01
	8	4.77	3.01
	12	-	-

Table 6. Hydrogen per oxide tolerance of *Lactobacillus* (log cfu/ml)

TIME	STRAINS	
	LAB VJ 15	LAB VJ 32
30 min	6.02	7.78
1	6.02	6.98
2	4.77	6.98
3	3.01	6.02
4	1	3.01
6	-	1

Table. 7. Sodium chloride tolerance of *Lactobacillus*

Concentration of NaCl (%)	STRAINS	
	LAB VJ 15	LAB VJ 32
1	0.815	0.926
2	0.625	0.731
3	0.372	0.485
4	0.116	0.173
5	0.083	0.085

Table. 8. Probiotic efficiency (Wg) for chicken

DAYS	WEIGHT OF CHICKS (gm)	
	Control	LAB VJ 32
0th	82 ± 2.82	84 ± 1.75
7th	125 ± 3.50	148 ± 1.38
14th	200.5 ± 1.53	238 ± 2.06
21st	254.5 ± 2.51	322 ± 2.51
28th	327.5 ± 2.08	402.5 ± 3.62
35th	400 ± 2.69	493 ± 2.18
42nd	477.5 ± 1.83	591 ± 2.42

Table. 9. Weight gain

Days (WEEKS)	Weight Gain (in gm)	
	CONTROL	LAB VJ 32
1	52.43	76.19
2	144.51	183.33
3	210.36	283.33
4	299.39	379.16
5	387.80	486.90
6	501.26	603.57

Table.10. FCR determination in poultry

DAYS (WEEK)	FCR	
	CONTROL	LAB VJ 32
1	5.33	4.12
2	5.17	5.52
3	8.09	6.55
4	9.84	10.46
5	12.64	13.27

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