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Comparative Study on Synbiotic Effect of Fermented Rice Bran by Probiotic Lactic Acid Bacteria *Lactobacillus casei* and Newly Isolated *Lactobacillus plantarum* B_2 in Wistar Rats

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Abstract

Rice bran contains dietary fiber that have a potency as prebiotic, and fermented by probiotic in colon to produce lactic acid and short chain fatty acid (SCFA). Synbiotic effect of fermented rice bran by *Lactobacillus plantarum* B_2 and *Lactobacillus casei* in Wistar rats (*Rattus norvegicus*) were investigated.

The aim of this research was to compare the synbiotic effect of fermented rice bran by probiotic lactic acid bacteria (*L. plantarum* B_2 and *L. casei*) in Wistar rats *(Rattus norvegicus)*.

Research methods is using a two month old Wistar rats (*Rattus norvegicus*) consisted of four groups (F0, F1, F2, F3) with five rats of each group were adapted for ten days by giving standard diet (AIN-93). Experimental diets were : standard diet of AIN 93 M (F0), standard diet with formulated rice bran media (F1), standard diet with fermented rice bran media by *L. casei* (F2) and standard diet with fermented rice bran media by *L. plantarum* B_2 (F3). Total bacteria, LAB, *Escherichia coli* and *Salmonella* of rats fecal were measured for 20 days. The caecum LAB and SCFA production were measured at the 20th day after feeding.

The results show that the newly isolated of *L. plantarum* B_2 in conformity with rice bran (F3) showed higher characteristics in comparison to the commercial *L. casei,* with fecal bacteria at the 20th day experiment were 10,93 log CFU g^{-1}, fecal LAB 7,76 log CFU g^{-1}, total *Escherichia coli* 4,48 log CFU g^{-1}, total *Salmonella* 4,22 log CFU g^{-1}, the caecum LAB viability 8,83 log CFU g^{-1}, SCFA total (acetic, propionic and butyric acids 11,05 and 11,49 mg g^{-1}, therefore the F3 formula has potential as symbiotic product.

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1. Introduction

Functional foods which develope rapidly and attract human being are probiotic and prebiotic. Finland food scientist produced and developed probiotic beverages based on oat bran, known as Yosa. This beverage is fermented by *Lactobacillus achidophilus* LA5 and *Bifidobacterium* Bb 12. Yosa is rich of dietary fiber and has a potency as synbiotic beverages.Salovara and Scharlin (2001) explained that synbiotic effect is the synergy between probiotic and prebiotic.

Rice bran is by product from rice milling which contains high nutrition and relatively rich of dietary fiber. Kahlon (1993) showed that rice bran contains about 22,9% of dietary fiber such as hemicellulose, arabinogalactan, arabinoxylan, xyloglycan, proteoglycan, arabinofuranoside and raffinose. Rice bran also contains antioxidants, such as oryzanol, tocoferol, tocotrienol, and ferulic acid. Its mean that this commodity has a potency to be applied as fermentation medium to produce novel probiotic beverages.

Indigenous lactic acid bacteria from rice bran, known as *Lactobacillus plantarum* B_2 was isolated by Zubaidah and Farida (2005). Zubaidah and Valentine (2006) found that *Lactobacillus plantarum* B_2 has the highest viability than eight indigenous lactic acid bacteria of rice bran. Viability test through *in vitro* showed that *Lactobacillus casei* has higher viability than *Lactobacillus acidophillus*. Zubaidah and Hudayah (2006) found that through *in vitro* test, both of indigenous and commercial probiotic isolate utilize dietary fiber in rice bran to produce lactic acid and short chain fatty acid (SCFA).

In vitro study of synbiotic effect have some limitations. Adjusment of pH condition and bile salt could not show the real condition of intestine. Dunne *et al.* (2001), showed that *in vitro* test could not reach the accurate condition within intestine. Some of the facts is the fluctuative of both stomach pH and bile salt concentration. This fluctuation is influenced by amount and type of food consumption. Jacobsen *et al*.(1999) explained that *in vitro* test can not used to predict the living *Lactobacillus* within intestine.

Synbiotic effect through *in vivo* showed that probiotic microorganism utilize prebiotic substrate and convert it into short chain fatty acids (SCFA), i.e. acetic acid, propionic acid and butyric acid. The formation of SCFA can decrease the intestine pH, inhibit the pathogenic microorganism within intestine and prevent the colon cancer. Karpinem (2003) showed that acetic acid and propionic acid have an important role in providing energy for the cell, while butyric acid can regenerate the intestine mucosa cell.

The aim of this research was to compare the synbiotic effect of fermented rice bran by probiotic lactic acid bacteria (*L. plantarum* B_2 and *L. casei*) in Wistar rats *(Rattus norvegicus)*.

2. Materials and Methods

Microorganisms and Growth Condition. Indigenous LAB, *Lactobacillus plantarum* B_2 was isolated from rice bran. This culture was cultivated in sterile MRS broth (MRSB) medium. *Lactobacillus casei* was taken from PAU Pangan dan Gizi, Gadjah Mada University). This culture also cultivated in sterile MRSB medium. Those bacteria then incubated at 37^{o}C for overnight (about 12 and 14 hours respectively). Total bacteria, LAB, *Escherichia coli*, and *Salmonella* were investigated by using NA (Nutrient Agar), MRS agar (MRSA), EMBA medium and BSA (Bismuth Sulphite Agar), respectively.

Rice Bran Medium for Fermentation. Rice bran were collected from rice milling of IR 64 variety. The rice bran was sieved at 60 mesh sieve, then it was stabilized at 121^{o}C for three minutes. The stabilized rice bran was packaged with plastic in vacum condition, then it was stored at freezer temperature (about -20^{o}C). Rice bran medium (12% w/v) was homogenized at 85^{0}C for ± 10 minutes. After homogenous, the medium

then sterilized at 121^{0}C for ± 15 minutes using autoclave.

Fermented Rice Bran by Probiotic LAB. The rice bran medium was inoculated by 2% (v/v) of probiotic starter (*L. plantarum* B_2 and *L. casei*). The medium then incubated at 37^{o}C for 12 hours respectively. Those fermented medium then analyzed including the LAB total (cfu ml^{-1}), pH, and total acids (% titritable acids) in 0 and after 12 hours of fermentation.

***In vivo* Experiments.** Two months old of Wistar rats (*Rattus novergicus*) were reached from Medical Faculty, Brawijaya University. Those rats (about ±250 g in weight) were adapted for ten days by giving standard diet *American Institute Of Nutrition*/AIN-93 (AIN, 1993). For *in vivo* experiment, the rats were feed *ad libitum*. They were divided into four groups (F0, F1, F2, F3) with five rats of each group. There were four diet formulas : standard diet of AIN 93 M (F0) as negative control, standard diet with formulated rice bran media (F1) as positive control, standard diet with fermented rice bran media by *L. casei* (F2) and standard diet with fermented rice bran media by *L. plantarum* B_2 (F3). The formulated and fermented rice bran dossage for each F1, F2, and F3 is about 0,5 ml. The rats weight, Lactic Acid Bacteria (LAB), pH, total acid, total bacteria, *Escherichia coli* and *Salmonella* of rats fecal were measured for 20 days. After 20 days experiment, the rats were killed to get the caecum. Then, LAB viability, pH, total acid and SCFA of caecum were analyzed.

SCFA Analysis. SCFA production was measured after 20 days experiment.The caecums were weight, and diluted in aquades (1:8), then it was centrifuged at 6.750 G for 15 minutes using refrigerated centifuge (4^{o}C temperature). The pellet was removed, then the supernatant was diluted in 25% metaphosphoric acid (4:1). After that, the mixture was centrifuged at 31.000 G for 30 minutes, 4^{o}C temperature. Quantification of SCFA profile by injected the supernatant into Gas Chromatography (GC-14B Shimadzu) GP 10%, sp 1200/1%, metaphosphoric acid 80/100 mesg chromosorb WAW, 130^{o}C column temperature, 220^{o}C injector temperature, 0,98 N_2 Gaseous pressure (Modification of Titgemeyer et al., 1991).

Data Analysis. The resulted data were analyzed using ANOVA (α=5%) and differential test BNT (α=5%). The best treatment was obtained using ranking method (Tabucanon, 1988).

3. Results and Discussion

Fermented rice bran. According to the result shown in Table 1, total LAB fermented rice bran using *L.casei* (F2) after 12 hours fermentation were 1,07x10^9 CFU ml^{-1}, total acidity 0,878%, and pH 4,41. Total LAB fermented rice bran using *L.plantarum* B2 (F3) after 12 hours fermentation, were 1,25x10^9 CFU ml^{-1}, total acidity 0,879%, and pH 4,38.

Table 1. Fermented Rice Bran Characteristics

Formula type	LAB total (CFU mL^{-1}) Fermentation time			Organic acid total (%) Fermentation time			pH Fermentation time		
	0	12	*	0	12	*	0	12	**
F1	0	0	0	0,1953	0,1968	0,0015	6,25	6,25	0
F2	4,1x10^7	1,07x10^9	1,03x10^9	0,2926	0,878	0,5854	5,84	4,41	1,43
F3	4,4x10^7	1,25x10^9	1,21x10^9	0,2928	0,879	0,5862	5,83	4,38	1,45

(*) increasing value, (**) decreasing value

The increasing of LAB total during fermentation means that LAB using the rice bran nutrient effectively. LAB total of F3 medium is higher than F2. Rice bran is natural habitat for *L. plantarum* B_2, that has some specific enzyme to utilize and metabolize the rice bran substrate to produce energy. Charalampopoulus *et al*. (2002) explained that indigenous microorganism is more adaptable and utilize the substrate more effective than non-indigenous one. During fermentation process, LAB will ferment glucose into organic acids and

decrease the pH of rice bran medium. Karppinen (2003) explained that fermentation process by lactic acid bacteria is showed by producing some organic acid. Based on the Charalampopoulus *et al*. (2002), lactic acid and acetic acid accumulation during LAB fermentation will decrease the pH of fermentation medium.

Fecal Analysis. Figure 1 showed total total bacteria, total LAB, *Escherichia coli* and *Salmonella* of rats fecal. Total bacteria of fecal for all groups at the beginning of experiment were ranging from 10,32 to 10,91 log CFU g^{-1}. This fecal bacteria was increasing after 20 days treatment, ranging from 10,61 to 10,93 log CFU g^{-1} (Fig 1a). Fecal LAB for all groups at the beginning experiment were ranging from 6,82 to 6,98 log CFU g^{-1}. The fecal LAB were increasing after 20 days treatment, ranging from 7,24 to 7,76 log CFU g^{-1} (Fig 1b). Fecal LAB for F1, F2 and F3 were increasing about 0,34-0,84 unit log CFU g^{-1}, however F0 (control) was decreasing about 0,27 unit log CFU g^{-1}. F3 has the highest fecal LAB total than all groups.

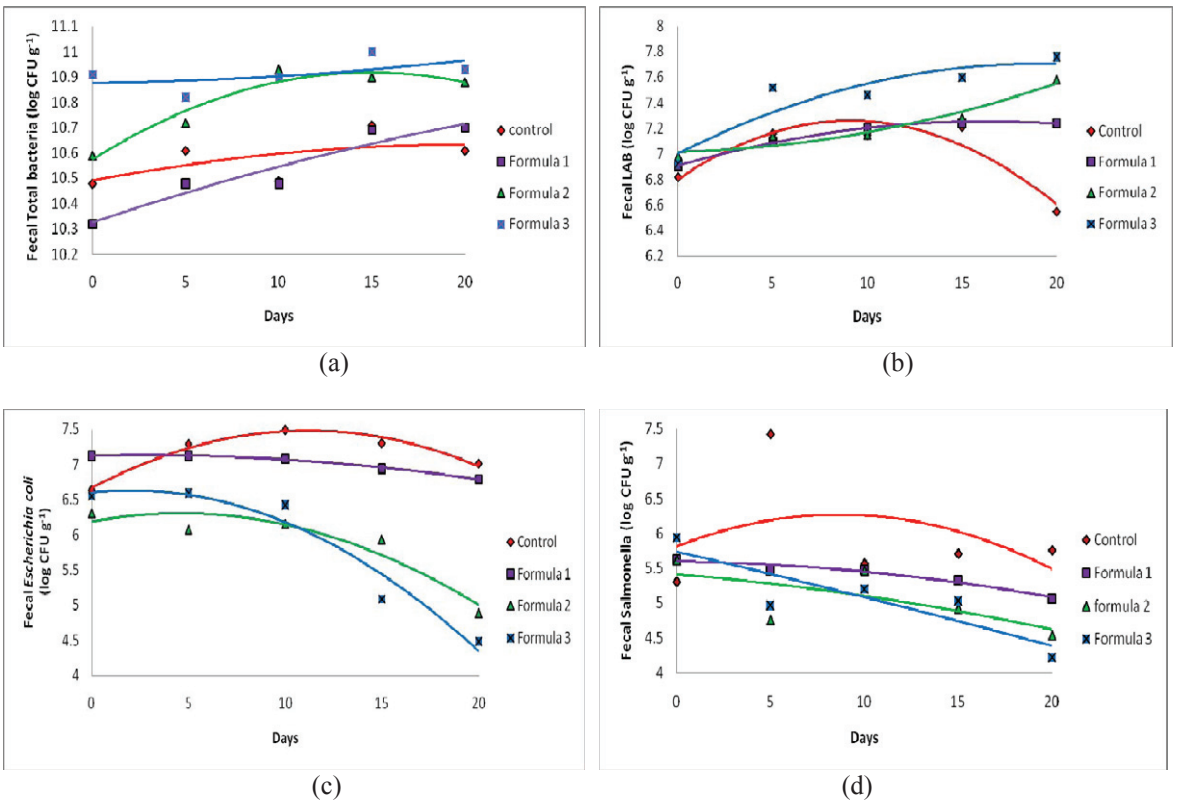

Fig. 1. Microbial Count (log counts CFU g^{-1}) in fresh feces of the Wistar Rats at the beginning of the experiment (d0) as well as after 5, 10, 15 and 20 d of consuming formulated diets. Fecal total bacteria (a), fecal LAB (b), fecal *Escherichia coli* (c), fecal *Salmonella* (d). Symbols : (♦)Control, (■)Formula 1, (▲)Formula 2, (x)Formula 3.

Figure 1 (c and d) showed the inhbition of pathogens (*Escherichia coli* and *Salmonella)* by probiotic microorganisms (F2 and F3). Fecal *Escherichia coli* for all groups at the beginning of experiment were ranging from 6,30 to 7,12 log CFU g^{-1}, then it were decreasing after 20 days treatment, ranging from 4,48 to 6,79 log CFU g^{-1} (Fig 1c). However F0 was increasing about 0,38 unit log CFU g^{-1}. Fecal *Escherichia coli* for F1, F2 and F3 were decreasing about 0,34 to 2,08 unit log CFU g^{-1}. Fecal *Salmonella* for all groups at the beginning of experiment were ranging from 5,31 to 5,94 log CFU g^{-1}, then it were decreasing after 20 days

treatment, ranging from 4,22 to 5,06 log CFU g^{-1} (Fig 1d). However F0 was increasing about 0,46 unit log CFU g^{-1}. Fecal *Salmonella* for F1, F2 and F3 were decreasing about 0,57 to 1,71 unit log CFU g^{-1}.

Lactic acid bacteria (LAB) produce organic acids such as lactate and acetate which acidify the surrounding at which the pathogenic organisms are unable to effectively to compete. Probiotic LAB viability within intestine showed that it have capability in degrading rice bran nutrient into simple molecule such as organic acids by colonic fermentation process. Vernazza *et al.* (2006), explained that various LAB produce antimicrobial peptide, which are secreted into growth medium. This peptide may inhibit the growth of enteropathogenic organisms such as enteropathogenic *Escherichia coli* and *Salmonella typhimurium*.

The highest inhibition to *Escherichia coli* and *Salmonella typhimurium* is F3 (fermented rice bran by *L. plantarum* B_2), which indigenous isolates of rice bran. Its indicated that natural microorganisms have good viability and activity in their original substrate. Zubaidah and Valentine (2006) explained that some probiotic isolates from rice bran is viable on extreme pH (2-3), bile salt concentration, and have antimicrobial activity through *in vitro* test. From those isolates, *L. plantarum* B_2 has the best characteristics than *L. casei*. Its mean that the result of probiotic viability by *in vitro* method is similar with *in vivo* test.

Caecum Analysis. Caecum is organ which has highly fermentation of carbohydrate, known as saccharolitic area. Fermentation process of dietary fiber (soluble and insoluble fiber) happened in caecum. Those dietary fiber then metabolize into organic acid.

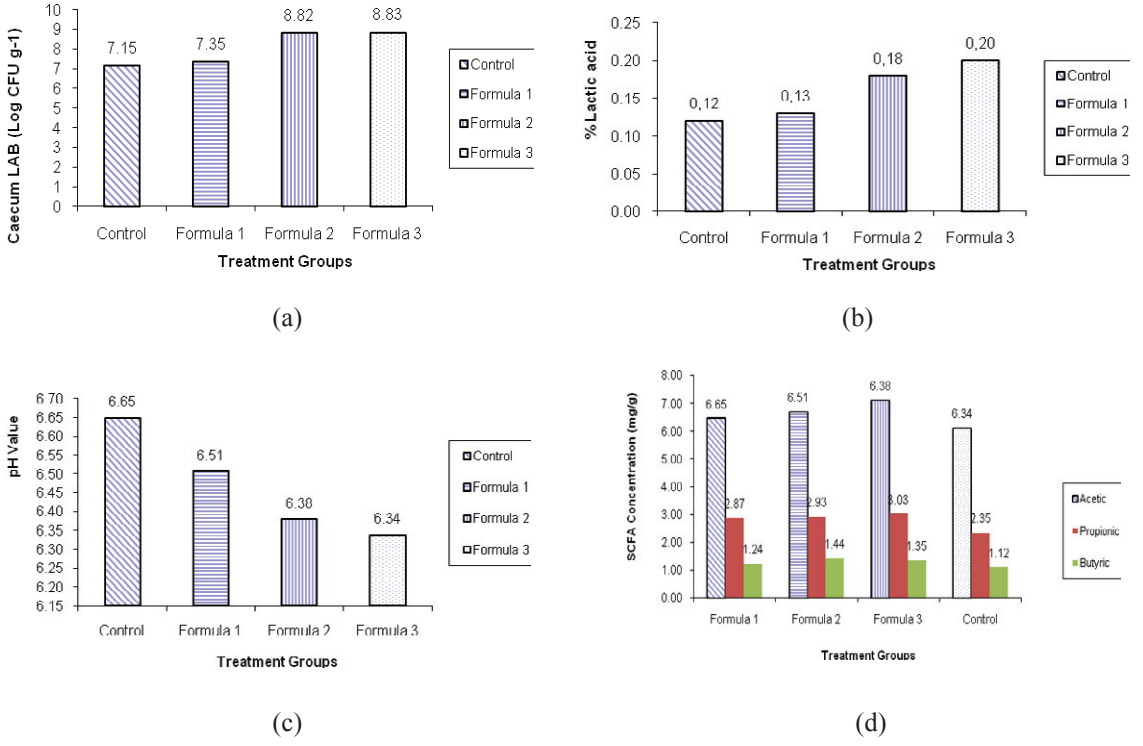

Fig 2. Caecum analysis after 20th days of formula feeding treatment: (a) LAB total, (b) Lactic acid total (the letter show significance difference at α=0.05), (c) pH value, (d) SCFA Concentration

Fig 2a showed the total LAB of caecum is ranging from 7,14 to 8,83 log CFU g^{-1}. F3 group has the highest LAB total than all goups. Lactic acid of caecum is ranging from 0,12-0,20% (Fig 2b). F3 has the highest lactic

acid, about 0,2%. Its caused by the highest LAB in F3 ($6,77x10^8$ CFU g^{-1}) and the lowest pH is F3 formula (F2c).

Dietary fiber of rice bran which have potency as prebiotic to promote LAB viability are raffinose and galactooligosaccharide (GOS). Probiotic will ferment prebiotic subtstrate into short chain fatty acid (SCFA) in the colon. Topping and Clifton (2001); Vernazza *et al.* (2006) explained that SCFA formation is produced in caecum.Tortuero (1997) described that raffinose can stimulate the *Lactobacillus* growth within colon. Richard *et al*., (2005) explained that combination or synergy between probiotic and prebiotic result synbiotic effect. Prebiotic fermentation by probiotic lactic acid bacteria will promote the benefit microorganisms within intestine and decrease the harmfull one.

Probiotic microorganisms of F2 formula (*L. plantarum* B_2) and F3 (*L. casei*) can stimulate indigenous microorganisms of colon, especially *Bifidobacterium* genus. Both of *L. plantarum* B_2 and *L. casei* are homofermentative lactic acid bacteria which produce lactic acid as main metabolite and SCFA as minor metabolite.

F3 has the highest SCFA concentration, approximately 11,49 mg g^{-1} (7,11 mg g^{-1} of acetic acid; 3,03 mg g^{-1} of propionic acid; and 1,35 mg g^{-1} of butyric acid (Fig. 2d). SCFA is produced from dietary fiber by intestine microorganisms (Miller and Wollin, 1996), unhydrolizable oligosaccharide (Mcfarlane et al., 1997). Vernazza *et al.* (2006) explained that the main fermentative substrates of dietary origin are nondigestible carbohydrate (e.g. resistant starch, non starch polysaccharides and fibres of plant origin and nondigestible oligosaccharides). Probiotic lactic acid bacteria have a good viability in the intestine. These bacteria will ferment prebiotic substrate into short chain fatty acid (SCFA) in the colon. SCFA can stimulate the regeneration of epithel cell of colon and have a potency to decrease the risk of colon cancer. Karppinen (2003) explained that *Bifidobacterium* is colonic bacteria which convert dietary fiber into lactic and asetic acid. The first step of fermentation process in colon is converting lactic and acetic acid. Second, this molecule convert into propionic and butyric acid. The conversion of organic acid is depend on the presence of dietary fiber in foods, the amount of fiber in colon and its microflora.

Zubaidah and Hudayah (2006) explained that SCFA concentration (acetic, propionic and butyric) of 48 hours fermented rice bran by *L. plantarum* B_2 were 8,75 mM, 5,75 mM dan 1,48 mM. This concentration is reached through *in vitro* test. Acetic acid is the highest concentration both *in vitro* and *in vivo* test. Duncan *et al.,* (2004) explained that colonic microorganisms of human and animal contain several bacteria which produce both of L- or D-lactic acid. This compound can be synthesize by *Lactobacillus*, *Bifidobacterium*, *Enterococcus* and *Eubacterium spp*.

SCFA production in human intestine is related to gas production, i.e. CO_2, CH_4, H_2. Cummings *et al.* (2001) showed that SCFA molar (acetic : propionic : butyric) of fermented wheat and oat bran is 64:16:20, whereas in fermented pectin (80:12:8), starch (62:15:23), non-starch polysaccharide (NSP) is 63:22:8.

Figure. 3d showed the highest SCFA molar is acetic acid, then followed by propionic and butyric acid. Wollin *et al.,* (1999), acetic acid is synthesize by glucose conversion into acetyl CoA then to pyruvic acid. Propionic conversion through two molecules : oxaloacetic and sucinic acid. 2 mol of acetyl CoA combined with 2 mol of pyruvic to produce butyric acid.

4. Conclusions

This study showed that fermented rice bran by *L. plantarum* B_2 performed higher ability synbiotic effect in LAB viability in both of fecal and caecum, inhibition to pathogenic bacteria (E.coli and salmonella), and SCFA production compare to F2 (fermented rice bran by *Lactobacillus casei*). In the future, the F3 formula can be applied as functional food (novel probiotic beverages).

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Appendix

Table 2. Ranking Method for Determination the Best Group

Rank	Group	Parameter								
		Δ Microbial Count in Fecal (log CFU g^{-1})				Fecal pH	Caecum			
		Total Bacteria	LAB	*E. coli*	Salmonella		LAB (log CFU g^{-1})	Organic acid (%)	pH	SCFA total (mg g^{-1})
1	F3	+ 0,02	+ 0,84	- 2,08	- 1,71	6,47	8,83	0,20	6,34	11,49
2	F2	+ 0,28	+ 0,60	- 1,41	- 1,07	6,50	8,82	0,18	6,38	11,05
3	F1	+ 0,38	+ 0,34	- 0,34	- 0,57	6,60	8,35	0,13	6,51	10,56
4	F0	+ 0,31	- 0,27	+ 0,38	+ 0,46	6,74	7,14	0,12	6,65	9,58

(Δ the 0 day and 20th days experiment) (+) increasing value from 0 to 20th days,(-) decreasing value from 0 to 20th days

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