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Synergy of antibacterial activity (*Salmonella thypimurium*) from probiotics and bioactive crude peptides from bekasam meat rabbit

Eka Wulandari, Andry Pratama, Wendry Setiyadi Putranto
Department of Livestock Product Technology, Animal Husbandry Faculty, Padjadjaran University, Sumedang, West Java, Indonesia

Abstract
Bekasam is a traditional Indonesian fermented animal food product. The use of rabbit meat as a raw material for besam is an effort to diversify processed functional food products from rabbit meat. This study aims to determine synergy activity antibacterial to bacteria S. thypimurium from probiotics and original peptides Bekasam meat rabbit. The research used experimental methods using a Completely Randomized Design. Statistical tests were carried out using variance tests and to determine differences between treatments, Duncan's multiple range test was carried out. The research results were obtained that there is synergy between *probiotics* and bioactive peptides in all treatment combinations (*in vitro*). From the results obtained, it was concluded that rabbit meat ekasam is a functional food product containing probiotics and bioactive peptides which synergize with each other at a combination of *probiotic concentrations* of 10^9 CFU/ mL and 5% peptides and can improve the immune system against *S. typhimurium*.

Keyword: Bioactive peptides, probiotics, Salmonella typhimurium, synergy

Introduction

Rabbit meat can be processed using the fermentation method, namely into bekasam. Bekasam is a traditional Indonesian fermented product. The microbes that are expected to live in the fermentation process of rabbit meat to become sour are Lactic Acid Bacteria (LAB). BAL is known to have the ability to survive and suppress the number of pathogenic bacteria in the human digestive tract. This ability is one of the properties possessed by bacteria that are classified as probiotics. Most types of LAB are included in probiotics.

Probiotics are live microorganisms that can have a positive impact on other organisms. Food ingredients that contain probiotics can be classified as a type of functional food. Nowadays, public awareness of consuming functional foods and the fact that probiotics in food provide health benefits has led to an increase in demand for food and drinks containing probiotics (Khartikeyan, *et al.*, 2014).

Bioactive peptides are defined as specific protein fragments that have a positive impact on body function or condition and can influence health (Kitts and Wiler, 2003). In the last decade, meat has been a source of bioactive peptides that are still being explored. Bioactive peptides in the form of parent protein are not yet active so the protein must be hydrolyzed first.

Bioactive peptides can be obtained in three ways, namely hydrolysis with digestive enzymes, protein hydrolysis by proteolytic microbes, hydrolysis by proteolytic enzymes produced by microbes (Brogden, 2005) [3]. Bioactive peptides have broad activities including antimicrobial, antioxidant, anticytotoxic, immunomodulatory, opioid and mineral transport activities (Korhonen and Pihlanto, 2006) [7].

Antimicrobial bioactive peptides work by interacting with the bacterial membrane which is then followed by membrane damage, disruption of membrane physiology such as cell wall biosynthesis, cell division or translocation across the membrane to interact with the cytoplasm of the target cell. It is generally assumed that the positive pole of the peptide interacts with the negative pole of the lipid on the outer surface or cytoplasmic membrane. Next, the peptide is inserted in a parallel position orientation in the bilayer into the inner part of the cytoplasmic membrane, which then results in the release of lipids. This will cause the lipids which are part of the cell membrane to experience changes in cell membrane permeability and membrane integrity resulting in cell lysis (Fjel, *et al,* 2012) [5]. Therefore _ it's on research This researched synergy from probiotics with the crude peptide obtained from Bekasam meat rabbit

Materials and Methods

The materials used in this research were 70% alcohol, selective medium NA (*Nutrien Agar*), MRSB medium, physiological NaCl, Mc Farland

In vitro testing uses the agar diffusion method *kirby -baur* to determine antimicrobial activity probiotics and bioactive peptides against *S. typhimurium.*

Test the inhibitory power of bacterial isolates against pathogenic bacteria using the *Kirby -mix disk method diffusion.* The pathogenic bacteria used *are Salmonella typhimurium* ATCC 14088. Pathogenic bacteria are diluted by mixing one cycle of the test bacterial culture into a test tube containing physiological NaCl and then homogenized using a vortex. The turbidity of the test solution was standardized using Mc solution Farland 0.5 which is equivalent to a bacterial count of $1.5\times^{108}$ / mL. Test bacteria that meet the standards are applied to the surface of the NA media. The paper discs were soaked in 20 µL LAB isolates grown in MRSB were then placed on the surface of NA media. Incubation was carried out at 37°C for 24 hours. Surrounding area _ The disc shows the sensitivity of the microbe to the peptide used as the test material expressed by the diameter of the clear zone. After incubation is complete, the diameter of the clear zone formed is measured using a ruler.

Discussion

Synergy Testing Probiotics and Peptide Extracts *In vitro*

Synergy testing *In vitro* probiotics and peptide extracts were carried out using the agar diffusion method which consisted of 3 test groups. The first group tested the inhibitory power of probiotics against *S. typhimurium,* the second group tested the inhibitory power of peptide extracts against *S. typhimurium* and the third group tested the combined inhibitory power of probiotics and peptide extracts against *S. typhimurium.*

Effect of Probiotics on the inhibition of S. typhimurium

The first group of tests on the effect of probiotics on the growth inhibition power of *S. typhimurium* is shown in Table 1. This test was carried out using 5 treatments, namely treatment P1 was a negative control where in the process the sample was only physiological NaCl, P2 was a positive control in the form of a pure isolate *of Bifidobacterium longum* which is used as the standard for positive results in this test, P3 is *Probiotic* 10^{7} CFU/ mL, P4 is *Probiotic* 10^{8} CFU/ mL and P5 is *Probiotic* 10^{9} CFU/ mL. *Bifidobacterium longum* was used as a standard because *B. longum* has been studied *in vitro* and *in vivo* to inhibit Salmonella infections (Slacanac, *et al.*, 2007 [11]; Silva, *et al.*, 2004) [10].

Table 1: Effect of Probiotics on Antimicrobial Activity *S. typhimurium In vitro*

Treatment /Repeat	**Obstacles zone**				
	X1	**X2**	**X3**	**X4**	**X5**
	----------------------mm--------------------------				
U1	0	15.3	13.8	15.2	15.5
U2	0	15.4	13.7	15.3	15.4
U3	0	15.3	13.8	15.1	15.6
U4	0	15.2	13.9	15.2	15.5
Average	0	15.3	13.8	15.2	15.5

Information

X1: Negative control (physiological NaCl)
X2: Positive control (*B. longum*)
X3: *Probiotics* 10^{7} CFU/ mL
X4: *Probiotics* 10^{8} CFU/ mL
X5: *Probiotics* 10^{9} CFU/ mL

Probiotic inhibitory power against *S. typhimurium* is in the range of 0-15.6 mm. To determine the effect of treatment on the administration of *S. typhimurium*, a Variety Test analysis was carried out. Based on the Variety Analysis analysis, it was found that the treatment had a real effect ($P<0.05$). To determine the differences between treatments, further analysis was carried out using the Duncan Test (Table 2).

Table 2: Duncan Test of the Effect of Probiotics on Antimicrobial Activity *S. typhimurium In vitro.*

Treatment	**Inhibitory Power Test Against *S. typhimurium* --------mm--------**	**Significance (0.05%)**
X1	0.0	a
X3	13.8	b
X4	15.2	b
X2	15.3	c
X5	15.5	d

Duncan test results in Table 2 at a *Probiotic* concentration of 10^{7} CFU/ mL (X3), the resulting inhibitory power value is not significantly different from the *Probiotic concentration* of 10^{8} CFU/ mL (X4) but both are significantly ($P<0.05$) higher than with negative control (X1). This illustrates that *probiotic treatment* starting at a concentration of 10^{7} CFU/ mL can inhibit the growth of *S. typhimurium.*

probiotic treatment with a concentration of 10^{9} CFU/ mL (X5) showed the greatest significantly different inhibitory power ($P<0.05$) compared to other treatments, especially the negative control and positive control. The difference in inhibitory power of the *probiotic concentration* used is due to the amount of antibacterial or bacteriocin produced by *the probiotic*, the more bacteria added, the greater the concentration of bacteriocin produced.

Probiotics have great inhibitory power against *S. typhimurium* because *L. buchneri* produces bacteriocins. *One of bacteriocin that is Buchnericin* has been studied inhibits the growth of gram-positive and gram-negative pathogenic bacteria, namely *Escherichia coli, Staphylococcus aureus, Salmonella typhi* and *Shigella flexneri* (Likaa Mahdi, *et al.*, 2017 [8]; Ayantola and Oladunmoye, 2016) [2]. Mechanism of antimicrobial activity *buchnericin* in inhibiting the growth of *S. typhimurium* is by damaging the permeability of cell membranes.

Hydrophobic residues on *Bacteriocins* experience direct contact with the cell membrane, then there is a change in cell membrane permeability and membrane integrity which causes cell lysis. Lysis that occurs due to the formation of holes in the cytoplasmic membrane is indicated by the activity of cell molecules moving in and out. The cell lysis that occurs has an impact on decreasing the gradient Cell pH. In general, the influence of the formation of cytoplasmic holes as a result of the presence of bacteriocins causes changes in the pH gradient and the release of intracellular molecules and the entry of extracellular metabolites (the environment). The effect causes cell growth to be inhibited and results in a death process in cells that are sensitive to bacteriocins (Drider, *et al.*, 2006) [4].

Effect of Peptides on the inhibitory power of S. typhimurium

The second group tested the effect of peptides on the growth inhibition power of *S. typhimurium,* using crude peptides.

Table 3: Effect of Peptides on Antimicrobial Activity *S. typhimurium In vitro*

Treatment/Repeat	**Obstacles zone**				
	Y1	**Y2**	**Y3**	**Y4**	**Y5**
	--------------------mm-------------------- ---				
U1	0	12.3	10.2	11.6	12.5
U2	0	12.4	10.3	11.7	12.6
U3	0	12.3	10.4	11.5	12.7
U4	0	12.2	10.3	11.6	12.2
Average	0	12.3	10.3	11.6	12.5

Information

Y1: Negative control (physiological NaCl)
Y2: Positive control (standard peptide)
Y3: 92% peptide
Y4: 95% peptide
Y5: 97% peptide

Data from Table 3 shows that the range of peptide inhibitory power against *S. typhimurium* is in the range of 0-12.7mm.

To determine the effect of treatment on the administration of *S. typhimurium*, a Variety Test analysis was carried out. Based on the Ragam Sidik analysis, it was found that the treatment had a real effect (p<0.05). To determine the differences between treatments, further analysis was carried out using the Duncan Test (Table 4).

Table 4: Duncan Test of the Effect of Peptides on Antimicrobial Activity *S. typhimurium In vitro*

Treatment	Inhibitory Power Test Against *S. Typhimurium* --------mm--------	Significance (0.05%)
Y1	0	a
Y3	10.3	b
Y4	11.6	c
Y2	12.3	d
Y5	12.5	e

The data in Table 4 shows that all treatments are significantly different from each other (P<0.05). The greater the peptide concentration used, the greater the antimicrobial activity produced. The greatest antimicrobial activity was obtained at 12.5 mm of Y5 (97% peptide concentration). This antimicrobial activity is also significantly different compared to the antimicrobial activity of the positive control, which means that the peptide isolated from rabbit meat has the potential to be synthesized and used as an antimicrobial compound in food ingredients.

The mechanism by which peptides can inhibit the growth of *S. typhimurium* is thought to be because peptides consisting of glutamic acid, glycine and isoleucine have hydrophobic groups which can interact with the lipid groups of lipopolysaccharide in the cell membrane producing hydrophobic interactions and also in acidic conditions the positively charged peptides will bind to the lipopolysaccharide. negatively charged which causes electrostatic interactions to occur. These two interactions will cause the cell membrane to be damaged and cause cell death.

Synergy Probiotics and Peptides against the inhibitory power of *S. typhimurium*

The third group of tests was testing the synergy effect of a combination of probiotics and peptides on the growth inhibition of *S. typhimurium.* the effect of probiotics on antimicrobial activity *S. typhimurium in vitro* is presented in Table 22. The data in Table 22 shows that the combination treatment of probiotics and peptides has greater antimicrobial activity compared to each treatment at the same concentration, treatment X1Y1 has antimicrobial activity of 1.0 4 mm (X1 = 13.8 mm and Y1 = 10.3mm), as well as for other combination treatments. So it can be concluded that there is positive synergy between probiotics and peptides at all treatment concentrations tested. The greatest antimicrobial activity was obtained in X3Y3 treatment (*L. buchneri* 109 ^{CFU} / mL and Peptide 97%).

Table 5: Testing the Synergy of the Effect of Probiotics and Peptides on Antimicrobial Activity *S. typhimurium In vitro*

Antimicrobial Activity Probiotics (mm)		Antimicrobial Activity of Peptides (mm)		Antimicrobial Activity Probiotics and Peptides (mm)	
X1	13.8	Y1	10.3	X1Y1	14.0
X2	15.2	Y2	11.6	X2Y2	15.6
X3	15.5	Y3	12.5	X3Y3	16.2
				X1Y3	14.2
				X3Y1	15.8

Information

X1: *L. buchneri* 10 ^{7} CFU/ mL
X2: *L. buchneri* 10 ^{8} CFU/ mL
X3: *L. buchneri* 10 ^{9} CFU/ mL
Y1: 92% peptide
Y2: 95% peptide
Y3: 97% peptide
X1Y1: *Probiotics* 10 ^{7} CFU/ mL and Peptides 92%
X2Y2: *Probiotics* 10 ^{8} CFU/ mL and Peptides 95%
X1Y3: *Probiotics* 10 ^{7} CFU/ mL and Peptides 97%
X3Y1: *Probiotics* 10 ^{9} CFU/ mL and Peptides 92%

The synergy between *probiotics* and peptides is thought to be because the bioactive peptides produced have prebiotic properties that support the growth of probiotic bacteria, this is in line with research by Arihara, *et al.* (2016) [1] who isolated the tripeptide (Glu -Leu- Met) which is prebiotic from hydrolyzate actomyosin and Lipke, *et al.* (2002) [9] obtained prebiotic peptides that supported the growth *of Bifidobacterium.*

Conclusion

The rabbit meat fermentation process produces probiotics and bioactive peptides which have antimicrobial activity. It was found that there was synergy between probiotics and bioactive peptides produced from the rabbit meat fermentation process with the best combination of 10 ^{9} CFU/ mL probiotics and 5% bioactive peptides for each dose used in *in vitro* testing.

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