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1 message

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Dear Professor Son Radu Editor in Chief of Food Research

We are pleased to submit an original research article entitled "Banana Resistant Starch inhibitory inflammation and Cyclooxygenase-2 in BALB/c Mice induced by Azoxymethane and Dextran Sodium Sulfate" by Syafira Noor Pratiwi, Diana Nur Afifah, Nyoman Suci Widyastiti, Vega Karlowee, Gemala Anjani, and Hermawan Istiadi.

In this manuscript, our research revealed that resistant starch from Indonesian local food, *batu* and *kepok* banana, can inhibit inflammation and supressed expression of COX-2. The treatment groups with resistant starch of batu and kepok banana flour had a significantly lower level of inflammation when compared to the positive group. The COX-2 score in the treatment group resistant starch of batu and kepok banana significantly lower level of significantly lower than the positive control group. The COX-2 intensity in both groups was lower than the positive group but not significant. The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups also had a lower than the positive control group.

We believe that this manuscript is appropriate for publication by the *Food Research* because it very related to aims and scope this journal. Our manuscript creates a paradigm for future studies of the evolution of functional food from Indonesian local food.

We confirm that this manuscript has not been published and is not under consideration for publication elsewhere. All authors have no conflict of interest that can affect the results of this study.

I will act as the author of the correspondence for this manuscript and I will be responsible for informing the progress or progress of the review of the manuscript, as well as revisions to all co-authors. For my correspondence, I can be contacted via email at d.nurafifah.dna@fk.undip.ac.id.

Thank you for your attention and consideration.

Best regards, Dr. Diana Nur Afifah, M.Si Nutrition Science Department Faculty of Medicine Diponegoro University Phone +6287770380468

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April 13rd, 2021

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I will act as the author of the correspondence for this manuscript and I will be responsible for informing the progress or progress of the review of the manuscript, as well as revisions to all co-authors. For my correspondence, I can be contacted via email at <u>d.nurafifah.dna@fk.undip.ac.id</u>.

Thank you for your attention and consideration.

Sincerely,

Dr. Diana Nur Afifah Doctor, Department of Nutrition Science Universitas Diponegoro



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[THERE IS NO NEED TO CHANGE THE FONT AND FONT SIZE] Banana Resistant Starch inhibitory inflammation and Cyclooxygenase-2 in BALB/c Mice induced by Azoxymethane and Dextran Sodium Sulfate

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22 Abstract

23 Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and 24 proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas 25 contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that induce by 26 Azoxymethane and Dextran Sodium Sulfate. This study main object was to determine the effect of 27 banana flour resistant starch on inflammation and Cox-2 in colon tissue. This research was experimental 28 with post-test only design involving 20 BALB/c mice in 4 groups, namely the negative control group, the positive control group, batu banana flour treatment and kepok banana flour treatment. The level of 29 30 inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 31 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light 32 microscope at a magnification of 400 times. The treatment groups with resistant starch of batu and 33 kepok banana flour had a significantly lower level of inflammation when compared to the positive group

- 34 (p = 0.035). The COX-2 score in the treatment group resistant starch of batu and kepok banana was
- 35 significantly lower than the positive control group (p<0.001). The COX-2 intensity in both groups was
- 36 lower than the positive group but not significant (p<0.001). The combined score between the
- 37 percentage and the intensity of COX-2 expression in the two treatment groups also had a lower than the
- positive control group (p<0.001). Resistant starch of batu and kepok banana can inhibit inflammation
- and supressed expression of COX-2.
- 40 Keywords: batu banana flour, colon cancer, kepok banana flour, resistant starch
- 41

42 1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030.(Birt and Phillips, 2014) The incidence of colon cancer would increase, especially in developing countries, including Indonesia.(Tiranda and Safitriana, 2018) It was estimated that Indonesians have a colon cancer risk of 5% or 1 in 10 Indonesians was estimated to have colon cancer.(Kemenkes RI, 2017) Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the etiology of colon cancer(Leu et al., 2007) and in one study determined that about 80% of colon cancer cases were related to diet.(Le Leu, Hu and Young, 2002) Those result

- 50 studies noted that colon cancer could be prevented.
- Resistant starch (RS) is an insoluble fiber that beneficial to human health including preventing colon cancer.(Tharanathan and Mahadevamma, 2003; Hovhannisyan, Aroutiounian and Glei, 2009; Purwanti and Suhartono, 2014) In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate.(Hu et al., 2016) Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained.(Augenlicht et al., 2002) Butyrate also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apontosic (Augenlicht et al., 2002) Wong et al., 2006)
- and increasing apoptosis.(Augenlicht et al., 2002; Wong et al., 2006)
- The source of RS is contained in Indonesian local fruit, namely bananas where the largest component of banana fruit is starch found in the pulp. Batu banana (Musa balbisiana Colla) and Kepok banana (Musa paradisiaca formatypica) were types of banana which had high resistant starch content of 39.5% and 27.7% respectively.(Musita, 2009) Method to increase the RS content is to make banana flour using the method enzymatic autoclaving-cooling autoclaving-cooling (AC-E-AC).(Fuentes-Zaragoza et al., 2010) Current studies had revealed that kepok banana flour obtained by the autoclaving-cooling
- enzymatic autoclaving-cooling (AC-E-AC) method could increase batu banana and kepok banana up to
 52.95% and 55.8% respectively.(Afifah et al., 2018)
- 67 The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This 68 inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2
- 69 overload associated with various cancers such as colon cancer. (Chandrasekharan and Simmons, no date)
- 70 COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation
- 71 and thromboxane produce.
- 72 Results of previous studies indicated that the expression of several genes related to inflammation
- r3 such as COX-2 decreased significantly in the provision of RS.(Hu et al., 2016) Butyrate produced by RS
- 74 may play directly to the reduction of COX-2(Jahns et al., 2011) by inhibiting COX-2 transcription
- r5 elongation. (Tong et al., 2005) In addition, a mechanism that might occur was butyrate affect

inflammatory mediators that play a role in transcriptional activation of COX-2.(Usami et al., 2008),(Jung

- 77 et al., 2005)
- 78 Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against
- the effects of resistant starch which is found in bananas have the possibility to protect the colon in
- 80 cancer induction effect. This study aimed to prove whether the resistant starch of batu banana flour and
- 81 kepok banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.
- 82

83 2. Materials and methods

- This research was a quasi-experimental research design post only with the control group design.
 Feed making was carried out at the Diponegoro University Integrated Laboratory, while animal
 rearing experimental animals conducted at the Inter-University Central Food and Nutrition
 Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was conducted for 11 weeks. This
 study had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of
 Medicine, Diponegoro University and obtain a letter of approval Ethical Clearance under number 33
 / EC / H / FK-UNDIP / IV / 2019.
- 91

92 2.1 Experimental animals

This study used male BALB / c mice aged 5 weeks weighing about 20 to 25 grams. Animals
acclimatized beforehand for 7 days and then divided into 4 groups randomly, namely the negative
control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93
feed without induction and without any treatment. C+ was given the standard feed AIN-93 and by
the induction of AOM and DSS. T1 and T2 was the group that was given AOM and DSS induction and
given feed that had been modified with banana flour batu and kepok respectively.

Animals were caged in a room that had air conditioning and had a naturally dark light settings.
 Feed and drink provided ad libitum. AOM induction was given as 10 mg/kg intraperitoneally. AOM
 was dissolved in Phosphate Buffer Solution (PBS) at a ratio of 1: 1. AOM injection taken once after
 acclimatization. Giving DSS 2% performed the following day for 7 days.

Batu and kepok bananas cleaned and washed with running water then peeled. Banana pulp thin 103 104 cut of about 2 mm. Banana slices dried in the sun for 3 days. Dried banana pieces that had been 105 crushed and then sieved with a 80 mesh sieve. Banana flour treated autoclaving at 121 ° C for 15 106 minutes and then cooling at 4 ° C for 24 hours. The pH of banana flour was adjusted with 0.2 M 107 acetate buffer and 2% of the pullulanase enzyme was added (v / w of banana flour) then incubated 108 at 40 °C for 12 hours at 150 rpm. The reaction was stopped by heating the suspension at 85 °C for 5 109 minutes. Banana flour wrapped in aluminum foil then treated autoclaving at 121 ° C for 15 minutes 110 and cooling 4 ° C for 24 hours.

- 111
- 112
- 113 2.2 Immunohistochemistry

Animals terminated by dislocation of cervical done quickly and sterile. Colon fragments were fixed in 10 % Neutral Buffer Formalin and embedded in paraffin blocks. The specimen on the paraffin block serially sectioned using rotary microtome. The sections were stained with hematoxylin eosin (HE) and immunostaining COX-2 was performed according to the manufacturer's recommendations from FineTest (Wuhan, China).

- 119 Observations inflammation and COX-2 in colon tissue was done using light microscope with a
- magnification of 400 times. Each colon tissue sample was captured 5 random images from 5
- different fields. Inflammation scoring was performed within 4 scores, negative, minimal, mild and
- moderate. The score was 0 or negative if there was no inflammation. The score was 1 or mild if
- inflammation infiltrates the mucosa. The score was 2 or moderate if inflammation infiltrates the
 mucosa and submucosa. The score was 3 or moderate if the inflammation infiltrates transmural
- 125 (infiltrates to the tunica intima).(Erben et al., 2014)
- 126 Immunohistochemical observations were carried out by estimating the percentage of cells stained
- and the staining intensity. Scores percentage stained of COX-2 was divided into 5 scores, the score
 was 0 if the estimated percentage of 0% to 5%; the score was 1 if the estimated percentage of 6% to
 25%; the score was 2 if the estimated percentage of 26% to 50%; the score was 3 if the estimated
 percentage of 51% to 75%; and the score was 4 if the estimated percentage of 76% to 100%. The
 intensity of staining had 3 scores, score 1 if the slight yellowing, score 2 when the brownish yellow
- and score 3 when brown.(Wu and Sun, 2015)

Final scores was the sum of the staining intensity score and the cell staining percentage score. The combination of the score would had 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the

- combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8).(Wu andSun, 2015)
- 138

139 2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The
 normality test was carried out by the Saphiro Wilk test. Bivariate analysis was performed using the
 Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal.
 Multivariate analysis was performed by performing a Post Hoc test to see which variables
 contributed to the differentiation value. The statistical value is significant if the p-value is less than
 0.05.

146

147 3. Results and discussion/Results

Figure 1 shows that image B (positive control) had the most inflammation among other images. Inflamed tissue in treatment 1 and 2 was not worse than the positive control but more than the negative control.

151Table 2 revealed that each group had a significant difference in inflammatory values (p-Value =1520.035). Positive control group had levels of inflammation that was the highest among the other153groups, while the negative control group had the lowest levels of inflammation.

154Table 2 revealed that the inflammation value of the positive control group was statistically155significantly different from the negative control group, both treatment groups where the mean156value of inflammation in the positive control group was higher than the other groups. The157inflammation value in the negative group did not had a significant difference to the both treatment158groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value159in the negative group.

160 Cells that had COX-2 showed a brownish yellow in the cytoplasm which could be seen in Figure
161 2. Categories COX-2 expression in all four groups could be seen in Table 2 where the positive control
162 group had the highest severity percentage which had the expression of COX-2 with the category of

- strongly positive as much as 84%. The both treatment groups had the lowest severity with a positivecategory percentage of 48% and 50% respectively.
- 165The results of the COX-2 percentage score in colon tissue after the intervention was there a166difference in the four groups. Table 4 revealed that the positive control group had the highest COX-2167percentage score compared to other groups while the treatment group 2 had the lowest COX-2168percentage score.
- 169 Table 4 revealed that the positive control group had a different COX-2 percentage score for both 170 treatment groups where positive control had the highest mean among the other groups. Negative 171 control had a different value against other groups where the COX-2 score in treatment 1 and 172 treatment 2 lower. Both treatment groups did not have any differences, so it could be said that the 173 administration of batu and kepok banana resistant starch had the same effect on the COX-2. 174 The results of COX-2 intensity between groups showed a difference between groups. However, the 175 inter-group mean showed that the positive control group had an intensity of COX-2 were highest when compared with other groups. Treatment group 1 had the lowest COX-2 intensity value among 176 177 the other.
- Test results of the further test showed that the positive control group had a different intensity value significantly with other groups where other groups had a COX-2 intensity value was lower. The negative control group had a different COX-2 intensity value from the treatment group 1 and had the same COX-2 intensity value as the treatment group 2 where both treatment groups COX-2 intensity values was lower in the negative control group. The treatment group 1 had no difference intensity value of COX-2 with 2 treatment groups.
- 184The results of the combined value of percentages with intensity of COX-2 between groups showed a185differences between groups. However, the inter-group mean showed that the positive control group186had a combined value percentage of the intensity of COX-2 were highest when compared to other187groups. The treatment group 1 had a combined value percentage value with the intensity of COX-2188that was lowest among the other groups.
- Further test results combined value of the percentage with the intensity of COX-2 showed that the positive control group has a different intensity value significantly with other groups where other groups had combined value of the percentage with the intensity of COX-2 was lower. Negative control had different values from other groups where the combined value of the percentage with the intensity of COX-2 in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences means the administration of banana and banana batu banana resistant starch had the same effect on the combined value of the percentage with the intensity of COX-2.
- 196 197

198 4. Discussion (Please omit if you are combining results and discussion)

199The above results indicate that resistant starch has a protective effect on AOM and DSS-induced200colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM201and DSS compounds where the level of inflammation is the same as the groups that are not202induced by AOM and DSS compounds.

203The results of this study support the study conducted by Ying Hu et al where the inflammation204score decreased in experimental animals that were induced by AOM DSS and given a diet205containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical206compounds play an important role in the early stages of colorectal carcinogenesis in which the

- inflammation may induce chronic immune response resulting in cellular proliferation and
 regeneration.(Mariani, Sena and Roncucci, 2014) If the immune response fails, cytokines, growth
 factors and cellular respiration products will continue to proliferate to repair the wound. This can
 lead to the accumulation of genetic errors and improper proliferation continuously.(Mariani, Sena
 and Roncucci, 2014)
- Chronic inflammation can speed up tumor formation.(Hu et al., 2016)Therefore, individuals with ulcerative colitis are at high risk of developing Colitis Associated Cancer (CAC). This is supported by previous research that shows that RS can prevent colitis and Colorectal Cancer (CRC).4.23–25 So it can be concluded that the hospital can also prevent CAC.
- 216 The results of this study are reinforced by the results of previous studies where the positive 217 control group that was given only a standard diet had a high inflammatory score and had the 218 number of bacteria from the genus Fusibacterium, Escherichia and Enterococcus which were also 219 associated with CRC in humans. (Feng et al., 2015) Administration of AOM which induces DNA 220 damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community 221 which contributes to tumor formation. (Zackular et al., 2013) The study also indicated a dynamic 222 change in the microbiota population in the initial response to AOM and DSS before signs of 223 macroscopic tumor formation emerged. (Zackular et al., 2013) The crucial role of microbiotic 224 dysbiosis is supported by research where experimental animals that do not experience dysbiosis do 225 not experience inflammation and colon cancer. (Vannucci et al., 2008) Hospital administration can 226 increase bacteria associated with RS fermentation such as Parabacteroides, Ruminococcus and 227 Bifidobacterium.(Hu et al., 2016)In addition, there is also an increase in bacteria which is not 228 directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing 229 inflammation or regeneration of the colonic mucosa.29.30
- 230 SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-231 protein coupled receptors (GPRs) or by inhibiting deactivated hibatu. (Sebastián and Mostoslavsky, 232 2014) GPR43 is expressed in intestinal epithelial cells and certain immune cells but the expression 233 of GPR43 in humans with CRC and colitis is low in expression.32.33 Previous studies have shown a 234 positive effect that a high-fiber diet activates GPR43 and is characterized by a rapid increase in 235 acetate. (Macia, 2015) Another study showed that RS data significantly increased the expression 236 activity of GPR43. This indicates that GPR43 activation may have a role in intestinal 237 homeostasis.(Hu et al., 2016)Acetate and propionate have a significant inverse correlation with 238 tumor occurrence. Acetate and propionate modulate Treg cell and immune function. The 239 interaction between acetate and propionate with GPR43 indicates an anti-inflammatory effect of 240 RS.35.36
- 241 The COX-2 expression in the positive control group was significantly higher than the COX-2 242 expression in the negative control group and the two treatment groups. The results of this study 243 are supported by previous studies that showed the expression of genes associated with 244 inflammation such as COX-2 decreased significantly in the group given RS. The increased expression 245 of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an 246 inflammatory microenvironment that can enhance tissue dysplasia. (Hu et al., 2016) RS triggers 247 major changes in colonic gene expression that inhibits inflammatory pathways and suppresses 248 immune responses.(Haenen et al., 2013)
- 249High COX-2 expression is the beginning of tumorigenesis.(Wu and Sun, 2015) This may be250because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response,

- 251
- inhibiting apoptosis of tumor cells, increasing cell proliferation, regulating the cell cycle, increasing
- tumor angiogenesis, increasing the expression of metalloproteinases in tumor cells and stimulating 252
- 253 the activation of precursor substances that are carcinogenic. .(Wu and Sun, 2015) The role of COX-2
- 254 is reinforced by previous studies where most colon cancers have high COX-2 expression resulting in
- 255 tumor angiogenesis, immune system damage and tumor invasion.(Brown and DuBois, 2005)
- 256
- 257

258 **Conflict of interest**

- 259 The authors declare no conflict of interest.
- 260

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372 Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES 373

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Table 1. Composition of experimental animal feed g/100 g

Component (g)	Mice Group				
Component (g)	К-	K +	P1	P2	
<i>Batu</i> banana flour	-	-	19	-	
<i>Kepok</i> banana flour	-	-	-	18	
Corn starch	46.57	46.57	46.57	46.57	
Protein (casein)	14	14	14	14	
Dectrin	15.5	15.5	5.5	5.5	
Sucrose	10	10	10	10	
Soybean oil	4	4	4	4	
Alpacel (fiber)	5	5	5	5	
Mineral mix	3.5	3.5	3.5	3.5	
Vitamin mix	1	1	1	1	
L-cystine	0.18	0.18	0.18	0.18	
Choline	0.25	0.25	0.25	0.25	

375

376 377

	Table 2. Inflammation after intervention			
Group Mean ± Standard Deviation		<i>p</i> -Value		
К-	1.32 ± 0.33			
K +	2.48 ± 0.50			
P1	1.56 ± 0.38	0.035 *		
P2	1.40 ± 0.47			

378 379

*: there is a significant difference

a, b, c: different notations in the same column indicate a significant difference

Table 3. The relationship between COX-2 expression with various treatments

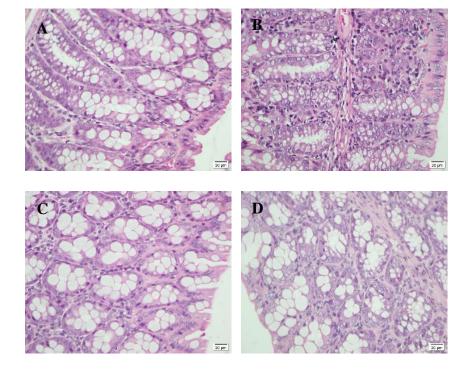
	COX-2					
Variable	n	Positive	Moderately positive	Strongly positive	p value	
Intervention					<0.001	
Control-negative	25	4 (16)	10 (40)	11 (44)		
AOM positive control	25	0 (0)	4 (16)	21 (84)		
AOM <i>batu</i> treatment	25	12 (48)	7 (28)	6 (24)		
AOM kepok treatment	20	10 (50)	9 (45)	1 (1)		

Table 4. Expression of C	COX-2 after	intervention
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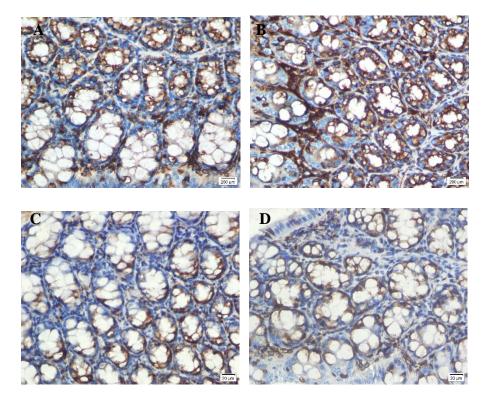
Group	COX-2 percentage	COX-2 intensity	Score and COX-2 intensity
	score		
K-	2.52 ± 0.92a	2.48 ± 0.51a	5.00 ± 1.32a
К +	3.52 ± 0.59b	2.76 ± 0.44b	6.28 ± 0.84b
P1	1.80 ± 1.32c	2.04 ± 0.74c	3.84 ± 1.86c
P2	1.40 ± 0.88c	2.35 ± 0.49ac	3.75 ± 1.12c
р	<0.001 *	<0.001 *	<0.001 *
*	*: there is a significant difference		

a h c: diffor

a, b, c: different notations in the same column indicate a significant difference



- 391 Figure 1.Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group,
- 392 magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification
- 393 400x; D: treatment group 2, magnification 400x.
- 394
- 395
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- 399
- 400 Figure 2.Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group,
- 401 magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification
- 402 400x; D: treatment group 2, magnification 400x.



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1 2	Banana Resistant Starch inhibitory inflammation and Cyclooxygenase-2 in BALB/c Mice induced by Azoxymethane and Dextran Sodium Sulfate
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18 Abstract

19 Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and 20 proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas 21 contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that induce by 22 Azoxymethane and Dextran Sodium Sulfate. This study main object was to determine the effect of 23 banana flour resistant starch on inflammation and Cox-2 in colon tissue. This research was experimental 24 with post-test only design involving 20 BALB/c mice in 4 groups, namely the negative control group, the 25 positive control group, batu banana flour treatment and kepok banana flour treatment. The level of 26 inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 27 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light 28 microscope at a magnification of 400 times. The treatment groups with resistant starch of batu and 29 kepok banana flour had a significantly lower level of inflammation when compared to the positive group 30 (p = 0.035). The COX-2 score in the treatment group resistant starch of batu and kepok banana was 31 significantly lower than the positive control group (p<0.001). The COX-2 intensity in both groups was 32 lower than the positive group but not significant (p<0.001). The combined score between the 33 percentage and the intensity of COX-2 expression in the two treatment groups also had a lower than the 34 positive control group (p<0.001). Resistant starch of batu and kepok banana can inhibit inflammation 35 and suppressed expression of COX-2.

- 36 Keywords: batu banana flour, colon cancer, kepok banana flour, resistant starch
- 37

38 1. Introduction

39 Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and 40 was expected to increase by 60% in 2030. (Birt and Phillips, 2014) The incidence of colon cancer would 41 increase, especially in developing countries, including Indonesia.(Tiranda and Safitriana, 2018) It was 42 estimated that Indonesians have a colon cancer risk of 5% or 1 in 10 Indonesians was estimated to have 43 colon cancer.(Kemenkes RI, 2017) Evidence from epidemiological and experimental studies suggested 44 diet was a crucial factor in the etiology of colon cancer(Leu et al., 2007) and in one study determined 45 that about 80% of colon cancer cases were related to diet.(Le Leu, Hu and Young, 2002) Those result 46 studies noted that colon cancer could be prevented.

47 Resistant starch (RS) is an insoluble fiber that beneficial to human health including preventing colon 48 cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan, Aroutiounian and Glei, 2009; Purwanti 49 and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in 50 the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate 51 (Hu et al., 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and 52 function of the colonic epithelium can be maintained (Augenlicht et al., 2002). Butyrate also has 53 chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and 54 increasing apoptosis (Augenlicht et al., 2002; Wong et al., 2006).

- The source of RS is contained in Indonesian local fruit, namely bananas where the largest component of banana fruit is starch found in the pulp. Batu banana (Musa balbisiana Colla) and Kepok banana (*Musa paradisiaca* formatypica) were types of banana which had high resistant starch content of 39.5% and 27.7% respectively.(Musita, 2009) Method to increase the RS content is to make banana flour using the method enzymatic autoclaving-cooling autoclaving-cooling (AC-E-AC).(Fuentes-Zaragoza et al., 2010) Current studies had revealed that kepok banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase batu banana and kepok banana up to
- 62 52.95% and 55.8% respectively.(Afifah et al., 2018)
- The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This
 inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2
 overload associated with various cancers such as colon cancer. (Chandrasekharan and Simmons, no date)
- 66 COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation
- 67 and thromboxane produce.

68 Results of previous studies indicated that the expression of several genes related to inflammation

- such as COX-2 decreased significantly in the provision of RS.(Hu et al., 2016) Butyrate produced by RS
- 70 may play directly to the reduction of COX-2(Jahns et al., 2011) by inhibiting COX-2 transcription
- elongation. (Tong et al., 2005) In addition, a mechanism that might occur was butyrate affect

inflammatory mediators that play a role in transcriptional activation of COX-2.(Usami et al., 2008),(Jung
 et al., 2005)

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas have the possibility to protect the colon in cancer induction effect. This study aimed to prove whether the resistant starch of batu banana flour and kepok banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

79 2. Materials and methods

This research was a quasi-experimental research design post only with the control group design.
Feed making was carried out at the Diponegoro University Integrated Laboratory, while animal
rearing experimental animals conducted at the Inter-University Central Food and Nutrition
Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was conducted for 11 weeks. This
study had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of
Medicine, Diponegoro University and obtain a letter of approval Ethical Clearance under number 33
/ EC / H / FK-UNDIP / IV / 2019.

88 2.1 Experimental animals

This study used male BALB / c mice aged 5 weeks weighing about 20 to 25 grams. Animals acclimatized beforehand for 7 days and then divided into 4 groups randomly, namely the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and without any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 was the group that was given AOM and DSS induction and given feed that had been modified with banana flour batu and kepok respectively.

Animals were caged in a room that had air conditioning and had a naturally dark light settings.
 Feed and drink provided ad libitum. AOM induction was given as 10 mg/kg intraperitoneally. AOM
 was dissolved in Phosphate Buffer Solution (PBS) at a ratio of 1: 1. AOM injection taken once after
 acclimatization. Giving DSS 2% performed the following day for 7 days.

99 Batu and kepok bananas cleaned and washed with running water then peeled. Banana pulp thin cut of about 2 mm. Banana slices dried in the sun for 3 days. Dried banana pieces that had been 100 crushed and then sieved with a 80 mesh sieve. Banana flour treated autoclaving at 121 ° C for 15 101 102 minutes and then cooling at 4 ° C for 24 hours. The pH of banana flour was adjusted with 0.2 M 103 acetate buffer and 2% of the pullulanase enzyme was added (v / w of banana flour) then incubated 104 at 40 °C for 12 hours at 150 rpm. The reaction was stopped by heating the suspension at 85 °C for 5 105 minutes. Banana flour wrapped in aluminum foil then treated autoclaving at 121 ° C for 15 minutes 106 and cooling 4 ° C for 24 hours.

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2.2 Immunohistochemistry

Animals terminated by dislocation of cervical done quickly and sterile. Colon fragments were fixed in 10 % Neutral Buffer Formalin and embedded in paraffin blocks. The specimen on the paraffin block serially sectioned using rotary microtome. The sections were stained with hematoxylin eosin (HE) and immunostaining COX-2 was performed according to the manufacturer's recommendations from FineTest (Wuhan, China).

- Observations inflammation and COX-2 in colon tissue was done using light microscope with a
 magnification of 400 times. Each colon tissue sample was captured 5 random images from 5
 different fields. Inflammation scoring was performed within 4 scores, negative, minimal, mild and
 moderate. The score was 0 or negative if there was no inflammation. The score was 1 or mild if
- inflammation infiltrates the mucosa. The score was 2 or moderate if inflammation infiltrates the
- 120 mucosa and submucosa. The score was 3 or moderate if the inflammation infiltrates transmural 121 (infiltrates to the tunica intima) (Erben et al. 2014)
- 121 (infiltrates to the tunica intima).(Erben et al., 2014)

- 122 Immunohistochemical observations were carried out by estimating the percentage of cells stained
- and the staining intensity. Scores percentage stained of COX-2 was divided into 5 scores, the score
- 124 was 0 if the estimated percentage of 0% to 5%; the score was 1 if the estimated percentage of 6% to
- 125 25%; the score was 2 if the estimated percentage of 26% to 50%; the score was 3 if the estimated
- percentage of 51% to 75%; and the score was 4 if the estimated percentage of 76% to 100%. The
 intensity of staining had 3 scores, score 1 if the slight yellowing, score 2 when the brownish yellow
- and score 3 when brown.(Wu and Sun, 2015)
- Final scores was the sum of the staining intensity score and the cell staining percentage score. The combination of the score would had 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8).(Wu and Sun, 2015)
- 135 2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The
 normality test was carried out by the Saphiro Wilk test. Bivariate analysis was performed using the
 Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal.
 Multivariate analysis was performed by performing a Post Hoc test to see which variables
 contributed to the differentiation value. The statistical value is significant if the p-value is less than
 0.05.

143 **3. Results**

134

142

Figure 1 shows that image B (positive control) had the most inflammation among other images.
Inflamed tissue in treatment 1 and 2 was not worse than the positive control but more than the
negative control.

147Table 2 revealed that each group had a significant difference in inflammatory values (p-Value =1480.035). Positive control group had levels of inflammation that was the highest among the other149groups, while the negative control group had the lowest levels of inflammation.

- 150Table 2 revealed that the inflammation value of the positive control group was statistically151significantly different from the negative control group, both treatment groups where the mean152value of inflammation in the positive control group was higher than the other groups. The153inflammation value in the negative group did not had a significant difference to the both treatment154groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value155in the negative group.
- 156 Cells that had COX-2 showed a brownish yellow in the cytoplasm which could be seen in Figure 157 2. Categories COX-2 expression in all four groups could be seen in Table 2 where the positive control 158 group had the highest severity percentage which had the expression of COX-2 with the category of 159 strongly positive as much as 84%. The both treatment groups had the lowest severity with a positive 160 category percentage of 48% and 50% respectively.
- The results of the COX-2 percentage score in colon tissue after the intervention was there a
 difference in the four groups. Table 4 revealed that the positive control group had the highest COX-2
 percentage score compared to other groups while the treatment group 2 had the lowest COX-2
 percentage score.

- 165 Table 4 revealed that the positive control group had a different COX-2 percentage score for both
- 166 treatment groups where positive control had the highest mean among the other groups. Negative
- 167 control had a different value against other groups where the COX-2 score in treatment 1 and
- 168 treatment 2 lower. Both treatment groups did not have any differences, so it could be said that the
- administration of batu and kepok banana resistant starch had the same effect on the COX-2.
 The results of COX-2 intensity between groups showed a difference between groups. However, the
 inter-group mean showed that the positive control group had an intensity of COX-2 were highest
- when compared with other groups. Treatment group 1 had the lowest COX-2 intensity value amongthe other.
- Test results of the further test showed that the positive control group had a different intensity value significantly with other groups where other groups had a COX-2 intensity value was lower. The negative control group had a different COX-2 intensity value from the treatment group 1 and had the same COX-2 intensity value as the treatment group 2 where both treatment groups COX-2 intensity values was lower in the negative control group. The treatment group 1 had no difference intensity value of COX-2 with 2 treatment groups.
- 180The results of the combined value of percentages with intensity of COX-2 between groups showed a181differences between groups. However, the inter-group mean showed that the positive control group182had a combined value percentage of the intensity of COX-2 were highest when compared to other183groups. The treatment group 1 had a combined value percentage value with the intensity of COX-2184that was lowest among the other groups.
- Further test results combined value of the percentage with the intensity of COX-2 showed that the positive control group has a different intensity value significantly with other groups where other groups had combined value of the percentage with the intensity of COX-2 was lower. Negative control had different values from other groups where the combined value of the percentage with the intensity of COX-2 in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences means the administration of banana and banana batu banana resistant starch had the same effect on the combined value of the percentage with the intensity of COX-2.
- 194 **4.** Discussion

195The above results indicate that resistant starch has a protective effect on AOM and DSS-induced196colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM197and DSS compounds where the level of inflammation is the same as the groups that are not198induced by AOM and DSS compounds.

199 The results of this study support the study conducted by Ying Hu et al where the inflammation 200 score decreased in experimental animals that were induced by AOM DSS and given a diet 201 containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical 202 compounds play an important role in the early stages of colorectal carcinogenesis in which the 203 inflammation may induce chronic immune response resulting in cellular proliferation and 204 regeneration.(Mariani, Sena and Roncucci, 2014) If the immune response fails, cytokines, growth 205 factors and cellular respiration products will continue to proliferate to repair the wound. This can 206 lead to the accumulation of genetic errors and improper proliferation continuously. (Mariani, Sena 207 and Roncucci, 2014)

- Chronic inflammation can speed up tumor formation.(Hu et al., 2016)Therefore, individuals with ulcerative colitis are at high risk of developing Colitis Associated Cancer (CAC). This is supported by previous research that shows that RS can prevent colitis and Colorectal Cancer (CRC).4.23–25 So it can be concluded that the hospital can also prevent CAC.
- 212 The results of this study are reinforced by the results of previous studies where the positive 213 control group that was given only a standard diet had a high inflammatory score and had the 214 number of bacteria from the genus Fusibacterium, Escherichia and Enterococcus which were also 215 associated with CRC in humans. (Feng et al., 2015) Administration of AOM which induces DNA 216 damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community 217 which contributes to tumor formation. (Zackular et al., 2013) The study also indicated a dynamic 218 change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumor formation emerged.(Zackular et al., 2013) The crucial role of microbiotic 219 220 dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer. (Vannucci et al., 2008) Hospital administration can 221 222 increase bacteria associated with RS fermentation such as Parabacteroides, Ruminococcus and 223 Bifidobacterium.(Hu et al., 2016)In addition, there is also an increase in bacteria which is not 224 directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing 225 inflammation or regeneration of the colonic mucosa.29.30
- 226 SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-227 protein coupled receptors (GPRs) or by inhibiting deactivated hibatu. (Sebastián and Mostoslavsky, 228 2014) GPR43 is expressed in intestinal epithelial cells and certain immune cells but the expression 229 of GPR43 in humans with CRC and colitis is low in expression.32.33 Previous studies have shown a 230 positive effect that a high-fiber diet activates GPR43 and is characterized by a rapid increase in 231 acetate.(Macia, 2015)Another study showed that RS data significantly increased the expression 232 activity of GPR43. This indicates that GPR43 activation may have a role in intestinal 233 homeostasis.(Hu et al., 2016)Acetate and propionate have a significant inverse correlation with 234 tumor occurrence. Acetate and propionate modulate Treg cell and immune function. The 235 interaction between acetate and propionate with GPR43 indicates an anti-inflammatory effect of 236 RS.35.36
- The COX-2 expression in the positive control group was significantly higher than the COX-2 237 238 expression in the negative control group and the two treatment groups. The results of this study 239 are supported by previous studies that showed the expression of genes associated with 240 inflammation such as COX-2 decreased significantly in the group given RS. The increased expression 241 of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an 242 inflammatory microenvironment that can enhance tissue dysplasia.(Hu et al., 2016) RS triggers 243 major changes in colonic gene expression that inhibits inflammatory pathways and suppresses 244 immune responses.(Haenen et al., 2013)
- High COX-2 expression is the beginning of tumorigenesis. (Wu and Sun, 2015) This may be
 because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response,
 inhibiting apoptosis of tumor cells, increasing cell proliferation, regulating the cell cycle, increasing
 tumor angiogenesis, increasing the expression of metalloproteinases in tumor cells and stimulating
 the activation of precursor substances that are carcinogenic. .(Wu and Sun, 2015) The role of COX-2
 is reinforced by previous studies where most colon cancers have high COX-2 expression resulting in
 tumor angiogenesis, immune system damage and tumor invasion.(Brown and DuBois, 2005)

254 **Conflict of interest**

255 The authors declare no conflict of interest.

256

257 Acknowledgments

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES

370

Table 1. Composition of experimental animal feed g/100 g

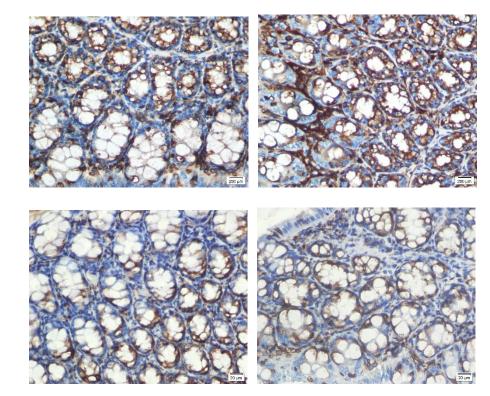
Component (a)	Mice Group							
Component (g)	К-	K +	P1	P2				
<i>Batu</i> banana flour	-	-	19	-				
<i>Kepok</i> banana flour	-	-	-	18				
Corn starch	46.57	46.57	46.57	46.57				
Protein (casein)	14	14	14	14				
Dectrin	15.5	15.5	5.5	5.5				
Sucrose	10	10	10	10				
Soybean oil	4	4	4	4				
Alpacel (fiber)	5	5	5	5				
Mineral mix	3.5	3.5	3.5	3.5				
Vitamin mix	1	1	1	1				
L-cystine	0.18	0.18	0.18	0.18				
Choline	0.25	0.25	0.25	0.25				

- 371
- 372

373	Table 2. Inflammation after intervention						
	Group	Mean ± Standard Deviation	<i>p</i> -Value				
	К-	1.32 ± 0.33					
	K +	2.48 ± 0.50					
	P1	1.56 ± 0.38	0.035 *				
	P2	1.40 ± 0.47					
374	*: there is a signifi	cant difference					
375	a, b, c: different n	otations in the same column indicate a	significant difference				
376							
377	Table 3. The relation	nship between COX-2 expression with va	arious treatments				

				COX-2			
Variable		n	Docitivo	Strongly	nyalua		
		n	Positive	positive	positive	p value	
Interven						<0.001	
	ontrol-negative	25	4 (16)	10 (40)	11 (44)		
	OM positive control	25	0 (0)	4 (16)	21 (84)		
	OM <i>batu</i> treatment	25	12 (48)	7 (28)	6 (24)		
A	OM <i>kepok</i> treatment	20	10 (50)	9 (45)	1 (1)		
		Expression	of COX-2 aft	er intervention			
Group	COX-2 percentage			COX-2 intensit	y Sco	ore and CO	X-2 intensit
	score			2.42 - 2.54		E 00 i	4.00
К-	2.52 ± 0.92a			2.48 ± 0.51a		5.00 ±	
K +	3.52 ± 0.59b			2.76 ± 0.44b		6.28 ±	
P1	1.80 ± 1.32c			2.04 ± 0.74c		3.84 ±	
P2	1.40 ± 0.88c			2.35 ± 0.49ac		3.75 ±	
p	<0.001 *			<0.001 *		<0.00)1 *
	: there is a significant diffe						
а	, b, c: different notations i	n the same	column indi	cate a significar	it difference	2	
1 111.50							
				20pm			
C							
Figure 1 Hem	atoxylin Eosin staining on t	he colonic	cell tissue of	mice <u>A</u> negati	ve control a	roup	
-	400x; B: positive control g			-	-	-	n
-	ment group 2, magnification				· ····································		

- 390
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- 395
- 396 Figure 2.Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group,
- 397 magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification
- 398 400x; D: treatment group 2, magnification 400x.



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Dear Professor Dr. Son Radu

Here I send the revised manuscript. I am sorry for the late reply because I need more time to revise the manuscript. Thank you.

Best regards, Dr. Diana Nur Afifah, M.Si Nutrition Science Department Faculty of Medicine Diponegoro University Phone +6287770380468

[Quoted text hidden]

2 attachments

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Evaluation Criteria	A (Excellent)	В	С	D	E (Worst)		
1. Appropriateness of Contents		х					
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5. Data Analysis			x				
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1.	Title It should reflect the article Follow Food Research format for title and author's names	The title and author's name had been change according to Food Research format		
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4.	Concise with sufficient background Lines 45-46, rewrite the sentence to remove the word "noted"	We had removed the word "noted"		
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	Clearly described and reproducible	We had rewritten the materials and methods		
	Rewrite the materials and methods marked in RED	marked in Red		
6.	Data Analysis			
	Results well presented and discussed Those section/sentences marked in RED in Results section must be rewritten in proper and correct ENGLISH	We had rewritten those section/sentence marked in Red in Results section to be in proper and correct ENGLISH		
	Must rewrite those marked in RED in discussion to be in proper and corre ct ENGLISH	We had rewritten those sentence marked in Red in Discussion section to be in proper and correct ENGLISH		
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	A clear summary of the study			



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1 2 3	Banana Resistant <u>resistant</u> Starch <u>starch</u> inhibitory inflammation and Cyclooxygenase<u>cyclooxygenase</u>- 2 in BALB/c Mice_mice induced by Azoxymethane<u>azoxymethane</u> and Dextran_dextran_Sodium_sodium Sulfatesulfate
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19 Abstract

20 Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and 21 proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas 22 contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that induce by 23 Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of bananas flour 24 toward inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice 25 in 4 groups, negative control group, positive control group, batu banana treatment group, and kepok 26 banana treatment group. This study main object was to determine the effect of banana flour resistant 27 starch on inflammation and Cox-2 in colon tissue. This research was experimental with post-test only 28 design involving 20 BALB/c mice in 4 groups, namely the negative control group, the positive control 29 group, batu banana flour treatment and kepok banana flour treatment_.-The level of inflammation was 30 seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 31 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification 32 of 400 times. The treatment groups with resistant starch of batu and kepok banana flour had a 33 significantly lower level of inflammation when compared to the positive group (p = 0.035). The COX-2 34 score in the treatment group resistant starch of batu and kepok banana was significantly lower than the 35 positive control group (p<0.001). The COX-2 intensity in both groups was lower than the positive group

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but not significant (p<0.001). The combined score between the percentage and the intensity of COX-2
expression in the two treatment groups also had a lower than the positive control group (p<0.001).
Resistant starch of batu and kepok banana can inhibit inflammation and suppressed expression of COX-2.

40 **Keywords:** *batu* banana flour, colon cancer, *kepok* banana flour, resistant starch

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42 1. Introduction

43 Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and 44 was expected to increase by 60% in 2030. (Birt and Phillips, 2014) The incidence of colon cancer would 45 increase, especially in developing countries, including Indonesia -(Tiranda and Safitriana, 2018). It was 46 estimated that Indonesians have a colon cancer risk of 5% or 1 in 10 Indonesians was estimated to have 47 colon cancer_r(Kemenkes RI, 2017). Evidence from epidemiological and experimental studies suggested 48 diet was a crucial factor in the etiology of colon cancer (Leu et al., 2007) and in one study determined 49 that about 80% of colon cancer cases were related to diet_-(Le Leu, Hu and Young, 2002). Those result 50 studies noted indicate that colon cancer could be prevented.

51 Resistant starch (RS) is an insoluble fiber that beneficial to human health including preventing colon 52 cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan, Aroutiounian and Gleiet al., 2009; 53 Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by 54 bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) 55 is butyrate (Hu et al., 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht et al., 2002). Butyrate 56 57 also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing 58 differentiation and increasing apoptosis (Augenlicht et al., 2002; Wong et al., 2006).

59 The source of RS is contained in Indonesian local fruit, namely-bananas where the largest 60 component of banana fruit is starch found in the pulp. Batu banana (Musa balbisiana Colla) and Kepok 61 banana (Musa paradisiaca formatypica) were types of banana which had high resistant starch content of 39.5% and 27.7% respectively -(Musita, 2009). Method to increase the RS content is to make banana 62 63 flour using the method enzymatic autoclaving-cooling autoclaving-cooling (AC-E-AC).(Fuentes-Zaragoza et al., 2010) Current studies had revealed that kepok banana flour obtained by the autoclaving-cooling 64 65 enzymatic autoclaving-cooling (AC-E-AC) method could increase batu banana and kepok banana up to 66 52.95% and 55.8% respectively.(Afifah et al., 2018) 67 The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 68 69 overload associated with various cancers such as colon cancer- (Chandrasekharan and Simmons, no 70 date2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet

aggregation and thromboxane produce.
 Results of previous studies indicated that the expression of several genes related to inflammation

such as COX-2 decreased significantly in the provision of RS_-(Hu *et al.*, 2016). Butyrate produced by RS

may play directly to the reduction of COX-2 (Jahns *et al.*, 2011) by inhibiting COX-2 transcription
 elongation.(Tong et al., 2005) In addition, a mechanism that might occur was butyrate affect

inflammatory mediators that play a role in transcriptional activation of COX-2 -(Usami *et al.*, 2008)-(;

77 Jung et al., 2005).

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Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against
 the effects of resistant starch which is found in bananas have the possibility to protect the colon in
 cancer induction effect. This study aimed to prove whether the resistant starch of batu banana flour and
 kepok banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

83 2. Materials and methods

This research was a quasi-experimental research design post only with the control group design.
 Animal food was made in the Feed making was carried out at the Diponegoro University Integrated
 Laboratory, while animal rearing eExperimental animals conducted were kept at the Inter University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The
 study was conducted done for 11 weeks. This study research had ethical approval from the Ethics
 Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a

90 letter of approval Ethical Clearance under number 33 / EC / H / FK-UNDIP / IV / 2019.

2.1 Experimental animals

93This study used male BALB / c mice aged 5 weeks weighing about 20 to 25 grams. Animals94acclimatized beforehand for 7 days and then divided into 4 groups randomly, namely the negative95control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-9396feed without induction and without any treatment. C+ was given the standard feed AIN-93 and by97the induction of AOM and DSS. T1 and T2 was the group that was given AOM and DSS induction and98given feed that had been modified with banana flour batu and kepok respectively.

Animals were caged in a room that had air conditioning and had a naturally dark light settings.
 Feed and drink provided ad libitum. They were injected by AOM induction was given as much as 10
 mg/kg intraperitoneally. AOM was dissolved in Phosphate Buffer Solution (PBS) at a ratio of 1: 1.
 AOM injection taken once after acclimatization. Giving-DSS 2% was givenperformed the following
 day for 7 days.
 Batu and kepok bananas cleanedwere-peeled and washed with by running water then peeled.

105 Banana were pulp thin slicedcut of about 2 mm. Banana slices then were dried in the sun for 3 days. 106 Dried banana pieces that had beenwere crushed and then were sieved with a 80 mesh sieve. Banana 107 flour were treated autoclaving at 121 ° C for 15 minutes and then coolingwere cooled at 4 ° C for 24 108 hours. The pH of banana flour was adjusted with 0.2 M acetate buffer and-then 2% of the 109 pullulanase enzyme was added (v / w of banana flour). then. The banana flour were incubated at 40 110 ° C for 12 hours at 150 rpm. The reaction was stopped by heating the suspension at 85-°-C for 5 111 minutes. Banana flour were wrapped in aluminum foil then treated were autoclaving at 121-°-C for 112 15 minutes and cooling were cooled at 4-°-C for 24 hours.

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2.2 Immunohistochemistry

116Animals_were terminated by dislocation of cervical done-quickly and sterile. The colon Colon117fragments were fixed in 10 % Neutral Buffer Formalin and embedded in paraffin blocks. The118specimen colon_on the paraffin block serially sectioned using rotary microtome. The colon_sections119were stained with hematoxylin eosin (HE)_and immunostaining_Immunostaining_COX-2 was_were120performed according to the manufacturer's recommendations from [Fine_Test_{Wuhan, China].

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121 Observations inflammation and COX-2 inin the observation of colon tissues was-were done using 122 light microscope with a magnification of 400 times magnification- Each colon tissue sample was 123 captured 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, negative, minimal, mild and moderate. Tthe score was 0 or negative if there was no 124 125 inflammation.-T; the score was 1 or mild if inflammation infiltrates the mucosa.-T; the score was 2 or 126 moderate if inflammation infiltrates the mucosa and submucosa-T: the score was 3 or moderate if 127 the inflammation infiltrates transmural (infiltrates to the tunica intima)_-(Erben et al., 2014). 128 Immunohistochemical observations were carried out by estimating the percentage of cells

stained and the staining intensity. Scores percentage stained of COX-2 was divided into 5 scores, the score was 0 if the estimated percentage of 0% to 5%; the score was 1 if the estimated percentage of 6% to 25%; the score was 2 if the estimated percentage of 26% to 50%; the score was 3 if the estimated percentage of 51% to 75%; and the score was 4 if the estimated percentage of 76% to 100%. The intensity of staining had 3 scores, score 1 if the slight yellowing, score 2 when the brownish yellow and score 3 when have brown colour-(Wu and Sun, 2015).

Final scores was the sum of the staining intensity score and the cell staining percentage score. The combination of the score would had 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8)-(Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The
 normality test was carried out by the Saphiro Wilk test. Bivariate analysis was performed using the
 Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal.
 Multivariate analysis was performed by performing a Post Hoc test to see which variables
 contributed to the differentiation value. The statistical value is significant if the p-value is less than
 0.05.

149 3. Results

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 Figure 1 shows that image B (positive control) had the most inflammation among other images.
 Tissue in treatment 1 and treatment 2 more inflamed than positive control but the negative control had the least amount of inflammation. Inflamed tissue in treatment 1 and 2 was not worse than the positive control but more than the negative control.

Table 2 revealed that each group had a significant difference in inflammatory values (p-Value =
 0.035). Positive control group had levels of inflammation that was the highest among the other
 groups, while the negative control group had the lowest levels of inflammation.

157Table 2 revealed that the inflammation value of the positive control group was statistically158significantly different from the negative control group, both treatment groups where the mean159value of inflammation in the positive control group was higher than the other groups. The160inflammation value in the negative group did not had a significant difference to the both treatment161groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value162in the negative group.

163Cells that had COX-2 showed a brownish yellow in the cytoplasm which could be seen in Figure1642. Categories COX-2 expression in all four groups could be seen in Table 2 where the positive control

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165		group had the highest severity percentage which had the expression of COX-2 with the category of	
166		strongly positive as much as 84%. The both treatment groups had the lowerst severity with a	
167		positive category percentage of 48% and 50% respectively.	
168		The results of the COX-2 percentage score in colon tissue after the intervention was there a	
169		difference in the four groups. Table 4 revealed that the positive control group had the highest COX-2	
170		percentage score compared to other groups while the treatment group 2 had the lowest COX-2	
171		percentage score.	
172		Table 4 revealed that the positive control group had a different COX-2 percentage score and	
173		intensity for both treatment groups where positive control had the highest mean among the other	
174		groups. Negative control had a different value against other groups where the COX-2 score in	
175		treatment 1 and treatment 2 lower. Both treatment groups did not have any differences, so it could	
176		be said that the administration of batu and kepok banana resistant starch had the same effect on	
177		the COX-2.	
178		The results of COX-2 intensity between groups showed a difference between groups. However,	
179		the inter-group mean showed that the positive control group had an intensity of COX-2 were highest	
180		when compared with other groups. Treatment group 1 had the lowest COX-2 intensity value among	
181		the other.	
182		Test results of the further test showed that the positive control group had a different intensity	
183		value significantly with other groups where other groups had a COX-2 intensity value was lower. The	
184		negative control group had a different COX-2 intensity value from the treatment group 1 and had	
185		the same COX-2 intensity value as the treatment group 2 where both treatment groups COX-2	
186		intensity values was lower in the negative control group. The treatment group 1 had no difference	
187		intensity value of COX-2 with 2 treatment groups.	
188		The results of the combined value of <u>between percentages with and intensity of COX-2</u> between *	_
189		groups showed a differences between groups. However, the inter-group mean showed that the	\backslash
190		positive Positive control group had the highest a combined value percentage of the intensity of COX-	
191		2 were highest when compared to other groups. <u>T</u> and the treatment group 1 had the lowest a)
192		combined value percentage value with the intensity of COX-2 that was lowest among the other	
193		groups. Both treatment groups did not have any differences in combined value means the	
194		administration of banana and banana batu banana resistant starch had the same effect.	
195		Further test results <mark>combined value of the percentage with the intensity of COX-2</mark>	
196		showed that the positive control group has a different intensity value significantly with other groups	
197		where other groups had combined value of the percentage with the intensity of COX-2 was lower.	
198		Negative control had different values from other groups where the combined value of the	
199		percentage with the intensity of COX-2 in treatment 1 and treatment 2 was lower. Both treatment	
200		groups did not have any differences means the administration of banana and banana batu banana	
201		resistant starch had the same effect on the combined value of the percentage with the intensity of	
202		COX-2.	
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205	4.	Discussion	
206		The above results indicate that resistant starch in <u>batu</u> and <u>kepok</u> banana flours has may have a	
207		protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit	

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Formatted: Font: Italic Formatted: Font: Italic inflammation due to the induction of AOM and DSS compounds where the level of inflammation is
 the same as the groups that are not induced by AOM and DSS compounds.

210 The results of this study support the study conducted by Ying Hu et al (2016) where the 211 inflammation score decreased in experimental animals that were induced by AOM DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or 212 213 chemical compounds play an important role in the early stages of colorectal carcinogenesis in which 214 the inflammation may induce chronic immune response resulting in cellular proliferation and 215 regeneration. - (Mariani, Sena and Roncucciet al., 2014). If the immune response fails, cytokines, 216 growth factors and cellular respiration products will continue to proliferate to repair the wound. 217 This can lead to the accumulation of genetic errors and improper proliferation.-continuously. 218 (Mariani, et al. Sena and Roncucci, 2014).

Chronic inflammation can speed up tumor formation.(Hu *et al.*, 2016)Therefore, individuals with
 ulcerative colitis are at high risk of developing Colitis Associated Cancer (CAC). This is supported by
 previous research that shows that RS can prevent colitis and Colorectal Cancer (CRC).
 4.23-25 So it
 can be concluded that the hospital can also prevent CAC.

223 The results of this study are reinforced by the results of previous studies where the positive 224 control group that was given only a standard diet had a high inflammatory score and had the 225 number of bacteria from the genus Fusibacterium, Escherichia and Enterococcus which were also 226 associated with CRC in humans_(Feng et al., 2015). Administration of AOM which induces DNA 227 damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community 228 which contributes to tumor formation_-(Zackular et al., 2013). The study also indicated a dynamic 229 change in the microbiota population in the initial response to AOM and DSS before signs of 230 macroscopic tumor formation emerged_-(Zackular et al., 2013). The crucial role of microbiotic 231 dysbiosis is supported by research where experimental animals that do not experience dysbiosis do 232 not experience inflammation and colon cancer_(Vannucci et al., 2008). Hospital administration can 233 increase bacteria associated with RS fermentation such as Parabacteroides, Ruminococcus and 234 Bifidobacterium_(Hu et al., 2016). In addition, there is also an increase in bacteria which is not 235 directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing 236 inflammation or regeneration of the colonic mucosa (Wong, et al., 2013; Nava and Stappenbeck, 237 <u>2011)</u>.29.30

238 SCFAs produced by the colonic microbiota -can stimulate cell function through activation of G-239 protein coupled receptors (GPRs) or by inhibiting deactivated hibatuhistone deacetylation 240 inhibition -(Sebastián and Mostoslavsky, 2014). GPR43 is GPR that expressed in intestinal epithelial 241 cells and certain immune cells, but the expression is lower when of GPR43 in humans withindividual 242 have CRC and colitis is low in expression condition (Tang, et al., 2011; Maslowski, et al., 2009). 32.33 243 Previous studies have had showed a positive effect that a of high-fiber diet that can increase 244 activates activation of GPR43, and is characterized by a rapid increase in acetate. (Macia, 2015). 245 Another study showed that RS data can increased the activation of GPR43 expression significantly 246 increased the expression activity of GPR43. This Those studies indicates that GPR43 activation may 247 have a role in intestinal homeostasis_-(Hu et al., 2016). Furthemore, Aacetate and propionate have 248 a significant inverse correlation with tumor occurrence. Acetate and propionate by modulate Treg 249 cell and immune function. The interaction between acetate and propionate with GPR43Those 250 indicates an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota 251 RS(Smith, et al., 2013; Fukuda, et al., 2011).35.36

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252 High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This may be 253 because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response, 254 inhibiting apoptosis of tumor cells, increasing cell proliferation, regulating the cell cycle, increasing 255 tumor angiogenesis, increasing the expression of metalloproteinases in tumor cells and stimulating 256 the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 257 was reinforced by previous studies where most colon cancers have high COX-2 expression and 258 resulting in tumor angiogenesis, immune system damage and tumor invasion (Brown and DuBois, 259 2005).

260 The-COX-2 expression in the positive control group was significantly higher than the COX-2 261 expression in the negative control group and the two treatment groups. The results of this study 262 are supported by previous studies that showed the expression of genes associated with 263 inflammation such as COX-2 decreased significantly in the group given RS. The increased expression 264 of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an 265 inflammatory microenvironment that can enhance tissue dysplasia_(Hu et al., 2016). RS triggers 266 major changes in colonic gene expression that inhibits inflammatory pathways and suppresses 267 immune responses-(Haenen et al., 2013).

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275 276

277 Conflict of interest

278 The authors declare no conflict of interest.

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- 391

Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES

Component (g)	Mice Group				
Component (g)	K-	K +	P1	P2	
<i>Batu</i> banana flour	-	-	19	-	
<i>Kepok</i> banana flour	-	-	-	18	
Corn starch	46.57	46.57	46.57	46.57	
Protein (casein)	14	14	14	14	
Dectrin	15.5	15.5	5.5	5.5	
Sucrose	10	10	10	10	
Soybean oil	4	4	4	4	
Alpacel (fiber)	5	5	5	5	
Mineral mix	3.5	3.5	3.5	3.5	
Vitamin mix	1	1	1	1	
L-cystine	0.18	0.18	0.18	0.18	
Choline	0.25	0.25	0.25	0.25	

Group	Mean ± Standard Deviation	<i>p</i> -Value
К-	1.32 ± 0.33	
K +	2.48 ± 0.50	
P1	1.56 ± 0.38	0.035 *
P2	1.40 ± 0.47	

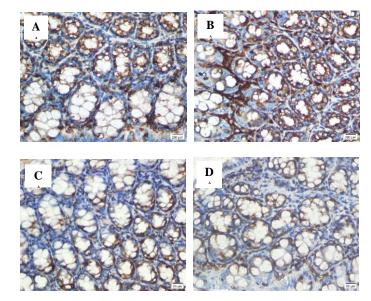
Table 3. The relationship between COX-2 expression with various treatments

a, b, c: different notations in the same column indicate a significant difference

			COX-2		
Variable	<u> </u>	Positive	Moderately	Strongly	p value
	n		positive	positive	
Intervention					<0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM batu treatment	25	12 (48)	7 (28)	6 (24)	
AOM kepok treatment	20	10 (50)	9 (45)	1 (1)	

06		Table 4. E	xpression of COX-2 after intervention	
	Group	COX-2 percentage	COX-2 intensity	Score and COX-2 intensity
-		score		
	K-	2.52 ± 0.92a	2.48 ± 0.51a	5.00 ± 1.32a
	K +	3.52 ± 0.59b	2.76 ± 0.44b	6.28 ± 0.84b
	P1	1.80 ± 1.32c	2.04 ± 0.74c	3.84 ± 1.86c
	P2	1.40 ± 0.88c	2.35 ± 0.49ac	3.75 ± 1.12c
	p	<0.001 *	<0.001 *	<0.001 *
7	*	*: there is a significant diffe	rence	
)8			n the same column indicate a significant diff	ference
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10 11				
	C		D	
12	100		tum	

- Figure 1._Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group,
- magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.



- Figure 2._Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification
- 400x; D: treatment group 2, magnification 400x.



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4 June 2021 at 02:13

Manuscript ID: FR-2021-262

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Please revise the manuscript according to the comments attached and revert to us as soon as possible. Adhering to Food Research format is greatly appreciated.

Best regards, Son Radu, PhD Chief Editor

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[Quoted text hidden]

FR-2021-262-revision.doc 1058K

1 2	Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate
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18 Abstract

19 Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and 20 proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that induce by 21 22 Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of bananas flour 23 toward inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice 24 in 4 groups, negative control group, positive control group, batu banana treatment group, and kepok 25 banana treatment group. The level of inflammation was seen from the colon tissue treated with 26 hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The 27 specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups 28 with resistant starch of batu and kepok banana flour had a significantly lower level of inflammation 29 when compared to the positive group (p = 0.035). The COX-2 score in the treatment group resistant 30 starch of batu and kepok banana was significantly lower than the positive control group (p<0.001). The 31 COX-2 intensity in both groups was lower than the positive group but not significant (p<0.001). The 32 combined score between the percentage and the intensity of COX-2 expression in the two treatment 33 groups also had a lower than the positive control group (p<0.001). Resistant starch of batu and kepok 34 banana can inhibit inflammation and suppressed expression of COX-2.

35 Keywords: batu banana, colon cancer, kepok banana, resistant starch

36

37 1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and 38 39 was expected to increase by 60% in 2030. (Birt and Phillips, 2014) The incidence of colon cancer would 40 increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was 41 estimated that Indonesians have a colon cancer risk of 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes RI, 2017). Evidence from epidemiological and experimental studies suggested 42 43 diet was a crucial factor in the etiology of colon cancer (Leu et al., 2007) and in one study determined 44 that about 80% of colon cancer cases were related to diet (Le Leu, Hu and Young, 2002). Those result 45 studies indicate that colon cancer could be prevented.

46 Resistant starch (RS) is an insoluble fiber that beneficial to human health including preventing colon 47 cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan, et al., 2009; Purwanti and Suhartono, 48 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will 49 produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu et al., 2016). 50 Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht et al., 2002). Butyrate also has chemoprotective 51 52 properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing 53 apoptosis (Augenlicht et al., 2002; Wong et al., 2006).

54 The source of RS is contained in Indonesian local fruit, bananas where the largest component of 55 banana fruit is starch found in the pulp. Batu banana (Musa balbisiana Colla) and Kepok banana (Musa 56 paradisiaca formatypica) were types of banana which had high resistant starch content of 39.5% and 57 27.7% respectively (Musita, 2009). Method to increase the RS content is to make banana flour using the 58 method enzymatic autoclaving-cooling autoclaving-cooling (AC-E-AC). (Fuentes-Zaragoza et al., 2010) 59 Current studies had revealed that kepok banana flour obtained by the autoclaving-cooling enzymatic 60 autoclaving-cooling (AC-E-AC) method could increase batu banana and kepok banana up to 52.95% and 61 55.8% respectively. (Afifah et al., 2018) 62 The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This 63 inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 64 overload associated with various cancers such as colon cancer (Chandrasekharan and Simmons, 2004). 65 COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation 66 and thromboxane produce. Results of previous studies indicated that the expression of several genes related to inflammation 67 68 such as COX-2 decreased significantly in the provision of RS (Hu et al., 2016). Butyrate produced by RS

may play directly to the reduction of COX-2 (Jahns *et al.*, 2011) by inhibiting COX-2 transcription
elongation.(Tong et al., 2005) In addition, a mechanism that might occur was butyrate affect
inflammatory mediators that play a role in transcriptional activation of COX-2 (Usami *et al.*, 2008; Jung *et al.*, 2005).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas have the possibility to protect the colon in cancer induction effect. This study aimed to prove whether the resistant starch of batu banana flour and kepok banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

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78 2. Materials and methods

This research was a quasi-experimental research design post only with the control group design.
Animal food was made in the the Diponegoro University Integrated Laboratory. Experimental
animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta,
Gadjah Mada University. The study was done for 11 weeks. This research had ethical approval from
the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and
obtain a letter of approval Ethical Clearance under number 33 / EC / H / FK-UNDIP / IV / 2019.

2.1 Experimental animals

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This study used male BALB / c mice aged 5 weeks weighing about 20 to 25 grams. Animals acclimatized beforehand for 7 days and then divided into 4 groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and without any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 was the group that was given AOM and DSS induction and given feed that had been modified with banana flour batu and kepok respectively.

Animals were caged in a room that had air conditioning and had a naturally dark light setting.
 They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization.
 DSS 2% was given the following day for 7 days.

96 Batu and kepok bananas were peeled and washed by water then peeled. Banana were sliced 97 about 2 mm then were dried in the sun for 3 days. Dried banana pieces were crushed and were 98 sieved with a 80 mesh sieve. Banana flour were treated autoclaving at 121 ° C for 15 minutes and 99 were cooled at 4 ° C for 24 hours. The pH of banana flour was adjusted with 0.2 M acetate buffer 100 then 2% of the pullulanase enzyme was added (v / w of banana flour). The banana flour were 101 incubated at 40 ° C for 12 hours at 150 rpm. The reaction was stopped by heating the suspension at 102 85°C for 5 minutes. Banana flour were wrapped in aluminum foil then were autoclaving at 121°C for 103 15 minutes and were cooled at 4°C for 24 hours.

2.2 Immunohistochemistry

Animals were terminated by dislocation of cervical quickly and sterile. The colons were fixed in
 Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin
 block serially sectioned using rotary microtome. The colon sections were stained with hematoxylin
 eosin (HE). Immunostaining COX-2 were performed according to the manufacturer's

111 recommendations (Fine Test, Wuhan, China).

in the observation of colon tissues were done using light microscope with 400 times magnification Each colon tissue sample was captured 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima) (Erben *et al.*, 2014).

118 Immunohistochemical observations were carried out by estimating the percentage of cells 119 stained and the staining intensity. Scores percentage stained of COX-2 was divided into 5 scores, the 120 score was 0 if the estimated percentage of 0% to 5%; the score was 1 if the estimated percentage of 121 6% to 25%; the score was 2 if the estimated percentage of 26% to 50%; the score was 3 if the Commented [acer5]: remove spacing
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estimated percentage of 51% to 75%; and the score was 4 if the estimated percentage of 76% to
100%. The intensity of staining had 3 scores, score 1 if the slight yellowing, score 2 when the
brownish yellow and score 3 when have brown colour (Wu and Sun, 2015).

Final scores was the sum of the staining intensity score and the cell staining percentage score. The combination of the score would had 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8) (Wu and Sun, 2015).

131 2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal.

- 135 Multivariate analysis was performed by performing a Post Hoc test to see which variables
- contributed to the differentiation value. The statistical value is significant if the p-value is less than0.05.

139 3. Results

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140Figure 1 shows that image B (positive control) had the most inflammation among other images.141Tissue in treatment 1 and treatment 2 more inflamed than positive control but the negative control142had the least amount of inflammation.

143Table 2 revealed that each group hada significant difference in inflammatory values (p-Value =1440.035). Positive control group had levels of inflammation that was the highest among the other145groups, while the negative control group had the lowest levels of inflammation.

146Table 2 revealed that the inflammation value of the positive control group was statistically147significantly different from the negative control group, both treatment groups where the mean148value of inflammation in the positive control group was higher than the other groups. The149inflammation value in the negative group did not had a significant difference to the both treatment150groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value151in the negative group.

152 Cells that had COX-2 showed a brownish yellow in the cytoplasm which could be seen in Figure 153 2. Categories COX-2 expression in all four groups could be seen in Table 2 where the positive control 154 group had the highest severity percentage which had the expression of COX-2 with the category of 155 strongly positive as much as 84%. The both treatment groups had lower severity with a positive 156 category percentage of 48% and 50% respectively.

Table 4 revealed that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 lower. Both treatment groups did not have any differences, so it could be said that the administration of batu and kepok banana resistant starch had the same effect on the COX-2.

164 The results of the combined value between percentages and intensity of COX-2 between groups 165 showed a differences. Positive control group had the highest combined value when compared to Commented [acer10]: The slight yellowing of?

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other groups and the treatment group 1 had the lowest combined value among the other groups.
 Both treatment groups did not have any differences in combined value means the administration of
 banana and banana batu banana resistant starch had the same effect.

170 4. Discussion

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The above results indicate that resistant starch in *batu* and *kepok* banana flours may have protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support the study conducted by Ying Hu et al (2016) where the 175 176 inflammation score decreased in experimental animals that were induced by AOM DSS and given a 177 diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or 178 chemical compounds play an important role in the early stages of colorectal carcinogenesis in which 179 the inflammation may induce chronic immune response resulting in cellular proliferation. (Mariani, 180 et al., 2014). If the immune response fails, cytokines, growth factors and cellular respiration 181 products will continue to proliferate to repair the wound. This can lead to the accumulation of 182 genetic errors and improper proliferation. (Mariani, et al., 2014).

183 The results of this study are reinforced by the results of previous studies where the positive 184 control group that was given only a standard diet had a high inflammatory score and had the 185 number of bacteria from the genus Fusibacterium, Escherichia and Enterococcus which were also 186 associated with CRC in humans (Feng et al., 2015). Administration of AOM which induces DNA 187 damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community 188 which contributes to tumor formation (Zackular et al., 2013). The study also indicated a dynamic 189 change in the microbiota population in the initial response to AOM and DSS before signs of 190 macroscopic tumor formation emerged (Zackular et al., 2013). The crucial role of microbiotic 191 dysbiosis is supported by research where experimental animals that do not experience dysbiosis do 192 not experience inflammation and colon cancer (Vannucci et al., 2008). Hospital administration can 193 increase bacteria associated with RS fermentation such as Parabacteroides, Ruminococcus and 194 Bifidobacterium (Hu et al., 2016). In addition, there is also an increase in bacteria which is not 195 directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing 196 inflammation or regeneration of the colonic mucosa (Wong, et al., 2013; Nava and Stappenbeck, 197 2011).

198 SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-199 protein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 200 2014). GPR43 is GPR that expressed in intestinal epithelial cells and certain immune cells, but the 201 expression is lower when individual have CRC and colitis condition (Tang, et al., 2011; Maslowski, et 202 al., 2009). Previous studies had showed a positive effect of high-fiber diet that can increase 203 activation of GPR43.(Macia, 2015). Another study showed that RS can increased the activation of 204 GPR43 expression significantly. Those studies indicates that GPR43 activation may have a role in 205 intestinal homeostasis (Hu et al., 2016). Furthemore, acetate and propionate have a significant 206 inverse correlation with tumor occurrence by modulate Treg cell and immune function. Those 207 indicates an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Smith, et al., 2013; Fukuda, et al., 2011). 208

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Commented [acer16]: Full stop. Please be diligent in format writting

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Commented [acer18]: No full stop before the citations. Commented [acer19]: revise 209 High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This may be 210 because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response, 211 inhibiting apoptosis of tumor cells, increasing cell proliferation, regulating the cell cycle, increasing 212 tumor angiogenesis, increasing the expression of metalloproteinases in tumor cells and stimulating 213 the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 214 was reinforced by previous studies where most colon cancers have high COX-2 expression and 215 resulting in tumor angiogenesis, immune system damage and tumor invasion (Brown and DuBois, 216 2005).

217 COX-2 expression in the positive control group was significantly higher than the COX-2 218 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with 219 220 inflammation such as COX-2 decreased significantly in the group given RS. The increased expression 221 of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an 222 inflammatory microenvironment that can enhance tissue dysplasia (Hu et al., 2016). RS triggers 223 major changes in colonic gene expression that inhibits inflammatory pathways and suppresses 224 immune responses (Haenen et al., 2013).

225 226

227 Conflict of interest

- 228 The authors declare no conflict of interest.
- 229

230 Acknowledgments

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- 247 Erben, U., Loddenkemper, C., Doerfel, K., Spieckermann, S., Haller, D., Heimesaat, M. M., Zeitz, M.,
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340

342 Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE

343 MANUSCRIPT BODY AFTER THE REFERENCES

Common ant (a)	Mice Group					
Component (g)	К-	K +	P1	P2		
<i>Batu</i> banana flour	-	-	19	-		
<i>Kepok</i> banana flour	-	-	-	18		
Corn starch	46.57	46.57	46.57	46.57		
Protein (casein)	14	14	14	14		
Dectrin	15.5	15.5	5.5	5.5		
Sucrose	10	10	10	10		
Soybean oil	4	4	4	4		
Alpacel (fiber)	5	5	5	5		
Mineral mix	3.5	3.5	3.5	3.5		
Vitamin mix	1	1	1	1		
L-cystine	0.18	0.18	0.18	0.18		
Choline	0.25	0.25	0.25	0.25		

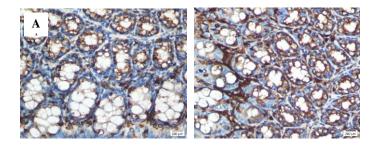
	Table 2. Inflammation after intervent	ion
Group	Mean ± Standard Deviation	<i>p</i> -Value
К-	1.32 ± 0.33	
K +	2.48 ± 0.50	
P1	1.56 ± 0.38	0.035 *
P2	1.40 ± 0.47	
*: there is a sig	nificant difference	

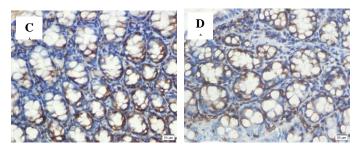
Table 3. The relationship between COX-2 expression with various treatments

a, b, c: different notations in the same column indicate a significant difference

	COX-2						
Variable	n Positive	Decitivo	Moderately	Strongly	nyalwa		
		positive	positive	p value			
Intervention					<0.001		
Control-negative	25	4 (16)	10 (40)	11 (44)			
AOM positive control	25	0 (0)	4 (16)	21 (84)			
AOM batu treatment	25	12 (48)	7 (28)	6 (24)			
AOM kepok treatment	20	10 (50)	9 (45)	1 (1)			

	Group	COX-2 percentage	Expression of COX-2 after intervention COX-2 intensity	Score and COX-2 intensity	
	Group	score	COX 2 Intensity	Score and Cox 2 intensit	
	K-	2.52 ± 0.92a	2.48 ± 0.51a	5.00 ± 1.32a	
	K +	3.52 ± 0.59b	2.76 ± 0.44b	6.28 ± 0.84b	
	P1	1.80 ± 1.32c	2.04 ± 0.74c	3.84 ± 1.86c	
	P2	1.40 ± 0.88c	2.35 ± 0.49ac	3.75 ± 1.12c	
	р	<0.001 *	<0.001 *	<0.001 *	
57	*	: there is a significant differ	rence		
8	a	, b, c: different notations ir	the same column indicate a significant diff	erence	
9					
60 61	A				
62	Ċ				
53	0	Figure 1. Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group,			
54		magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification			
65	400x · D· treat	ment group 2, magnificatio	n 400x.		





- 371
- 372 Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group,
- 373 magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification
- 374 400x; D: treatment group 2, magnification 400x.



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Dear Prof. Son Radu

I already revised the manuscript based on the Food Research format. Here is the revised manuscript. Thank you.

Best regards, Dr. Diana Nur Afifah, M.Si Nutrition Science Department Faculty of Medicine Diponegoro University Phone +6287770380468

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1 2	Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate
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18 Abstract

19 Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and 20 proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that induce by 21 22 Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of bananas flour 23 toward inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice 24 in 4 groups, negative control group, positive control group, batu banana treatment group, and kepok 25 banana treatment group. The level of inflammation was seen from the colon tissue treated with 26 hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The 27 specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups 28 with resistant starch of batu and kepok banana flour had a significantly lower level of inflammation 29 when compared to the positive group (p = 0.035). The COX-2 score in the treatment group resistant 30 starch of batu and kepok banana was significantly lower than the positive control group (p<0.001). The 31 COX-2 intensity in both groups was lower than the positive group but not significant (p<0.001). The 32 combined score between the percentage and the intensity of COX-2 expression in the two treatment 33 groups also had a lower than the positive control group (p<0.001). Resistant starch of batu and kepok 34 banana can inhibit inflammation and suppressed expression of COX-2.

35 Keywords: batu banana, colon cancer, kepok banana, resistant starch

36

37 1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and 38 39 was expected to increase by 60% in 2030. (Birt and Phillips, 2014) The incidence of colon cancer would 40 increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was 41 estimated that Indonesians have a colon cancer risk of 5% or 1 in 10 Indonesians was estimated to have 42 colon cancer (Kemenkes R, 2017). Evidence from epidemiological and experimental studies suggested 43 diet was a crucial factor in the etiology of colon cancer (Leu et al., 2007) and in one study determined 44 that about 80% of colon cancer cases were related to diet (Le Leu, Hu and Young, 2002). Those result 45 studies indicate that colon cancer could be prevented.

46 Resistant starch (RS) is an insoluble fiber that beneficial to human health including preventing colon 47 cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan, et al., 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will 48 49 produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu et al., 2016). 50 Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the 51 colonic epithelium can be maintained (Augenlicht et al., 2002). Butyrate also has chemoprotective 52 properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing 53 apoptosis (Augenlicht et al., 2002; Wong et al., 2006).

54 The source of RS is contained in Indonesian local fruit, bananas where the largest component of 55 banana fruit is starch found in the pulp. Batu banana (Musa balbisiana Colla) and Kepok banana (Musa 56 paradisiaca formatypica) were types of banana which had high resistant starch content of 39.5% and 57 27.7% respectively (Musita, 2009). Method to increase the RS content is to make banana flour using the 58 method enzymatic autoclaving-cooling autoclaving-cooling (AC-E-AC). (Fuentes-Zaragoza et al., 2010) 59 Current studies had revealed that kepok banana flour obtained by the autoclaving-cooling enzymatic 60 autoclaving-cooling (AC-E-AC) method could increase batu banana and kepok banana up to 52.95% and 61 55.8% respectively (Afifah et al., 2018). 62 The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This 63 inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 64 overload associated with various cancers such as colon cancer (Chandrasekharan and Simmons, 2004). 65 COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation 66 and thromboxane produce. Results of previous studies indicated that the expression of several genes related to inflammation 67 68 such as COX-2 decreased significantly in the provision of RS (Hu et al., 2016). Butyrate produced by RS

may play directly to the reduction of COX-2 (Jahns *et al.*, 2011) by inhibiting COX-2 transcription
elongation.(Tong et al., 2005) In addition, a mechanism that might occur was butyrate affect
inflammatory mediators that play a role in transcriptional activation of COX-2 (Usami *et al.*, 2008; Jung *et al.*, 2005; Usami *et al.*, 2008).
Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against

the effects of resistant starch which is found in bananas have the possibility to protect the colon in
 cancer induction effect. This study aimed to prove whether the resistant starch of batu banana flour and
 kepok banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

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78 2. Materials and methods

This research was a quasi-experimental research design post only with the control group design.
Animal food was made in the the Diponegoro University Integrated Laboratory. Experimental
animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta,
Gadjah Mada University. The study was done for 11 weeks. This research had ethical approval from
the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and
obtain a letter of approval Ethical Clearance under number 33 / EC / H / FK-UNDIP / IV / 2019.

2.1 Experimental animals

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This study used male BALB / c mice aged 5 weeks weighing about 20 to 25 grams. Animals acclimatized beforehand for 7 days and then divided into 4 groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and without any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 was the group that was given AOM and DSS induction and given feed that had been modified with banana flour batu and kepok respectively.

Animals were caged in a room that had air conditioning and had a naturally dark light setting.
 They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization.
 DSS 2% was given the following day for 7 days.

96 Batu and kepok bananas were peeled and washed by water then peeled. Banana were sliced 97 about 2 mm then were dried in the sun for 3 days. Dried banana pieces were crushed and were 98 sieved with a 80 mesh sieve. Banana flour were treated autoclaving at 121--C for 15 minutes and 99 were cooled at 4-°-C for 24 hours. The pH of banana flour was adjusted with 0.2 M acetate buffer 100 then 2% of the pullulanase enzyme was added (v / w of banana flour). The banana flour were 101 incubated at 40 ° C for 12 hours at 150 rpm. The reaction was stopped by heating the suspension at 102 85°C for 5 minutes. Banana flour were wrapped in aluminum foil then were autoclaving at 121°C for 103 15 minutes and were cooled at 4°C for 24 hours.

2.2 Immunohistochemistry

107Animals were terminated by dislocation of cervical quickly and sterilesterilized. The colons were108fixed in 10 % Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the109paraffin block serially sectioned using rotary microtome. The colon sections were stained with110hematoxylin eosin (HE). Immunostaining COX-2 were performed according to the manufacturer's111recommendations (Fine Test, Wuhan, China).

112 In-In_the observation of colon tissues were done using light microscope with 400 times 113 magnification_Each colon tissue sample was captured 5 random images from 5 different fields. 114 Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no 115 inflammation; the score was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or 116 moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the 117 inflammation infiltrates transmural (infiltrates to the tunica intima) (Erben *et al.*, 2014).

118 Immunohistochemical observations were carried out by estimating the percentage of cells 119 stained and the staining intensity. Scores percentage stained of COX-2 was divided into 5 scores, the 120 score was 0 if the estimated percentage of 0% to 5%; the score was 1 if the estimated percentage of 121 6% to 25%; the score was 2 if the estimated percentage of 26% to 50%; the score was 3 if the Commented [acer5]: remove spacing
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122	estimated percentage of 51% to 75%; and the score was 4 if the estimated percentage of 76% to		
123	100%. The intensity of staining had 3 scores, score 1 if the <u>tissue have</u> slight yellowing yellow colour,		Commented [acer10]: The slight yellowing of?
124	score 2 when the brownish yellow and score 3 when have brown colour (Wu and Sun, 2015).		
125	Final scores was the sum of the staining intensity score and the cell staining percentage score.		
126	The combination of the score would had 4 meaning, negative (if the value of the combination is 1 to		
127	2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the		
128	combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8) (Wu and		
129	Sun, 2015).		
130			
131	2.3 Data analysis		
132	Univariate analysis was performed to calculate the mean value and standard deviation. The		
133	normality test was carried out by the Saphiro Wilk test. Bivariate analysis was performed using the		
134	Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal.		
135	Multivariate analysis was performed by performing a Post Hoc test to see which variables		
136	contributed to the differentiation value. The statistical value is significant if the p-value is less than		
137	0.05.		
138			
139 3	. Results		
140	Figure 1 shows that image B (positive control) had the most inflammation among other images.		
141	Tissue in treatment 1 and treatment 2 more inflamed than positive control but the negative control		
142	had the least amount of inflammation. <u>Table 2 revealed that each group had a significant difference</u>		Commented [acer11]: Combine, paragraph needs to be
143	in inflammatory values (p-Value = 0.035). Positive control group had levels of inflammation that was		more than 3 lines
144	the highest among the other groups, while the negative control group had the lowest levels of		
145	inflammation.		
146			
147	Table 2 revealed that each group had a significant difference in inflammatory values (p-Value =+		Commented [acer12]: Combine, paragraph needs to be
148	0.035). Positive control group had levels of inflammation that was the highest among the other	$\overline{\ }$	more than 3 lines
149	groups, while the negative control group had the lowest levels of inflammation.		Formatted: Indent: First line: 0"
150	Table 2 revealed Furthermore, that the inflammation value of the positive control group was		Commented [acer13]: Add a continuation term, as this is
151	statistically significantly different from the negative control group, both treatment groups where the		about the same
152	mean value of inflammation in the positive control group was higher than the other groups. The		
153	inflammation value in the negative group did not had a significant difference to the both treatment		
154	groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value		
155	in the negative group.		
156	Cells that had COX-2 showed a brownish yellow in the cytoplasm which could be seen in Figure		
157	2. Categories COX-2 expression in all four groups could be seen in Table 2 where the positive control		
158	group had the highest severity percentage which had the expression of COX-2 with the category of		
159	strongly positive as much as 84%. The beneficial benefi		Commented [acer14]: Remove 'the '
160	category percentage of 48% and 50% respectively.		
161	Table 4 revealed that the positive control group had a different COX-2 percentage score and		
100			

intensity for both treatment groups where positive control had the highest mean among the other

groups. Negative control had a different value against other groups where the COX-2 score in

treatment 1 and treatment 2 lower. Both treatment groups did not have any differences, so it could

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be said that the administration of batu and kepok banana resistant starch had the same effect onthe COX-2.

168 The results of the combined value between percentages and intensity of COX-2 between groups 169 showed a differences. Positive control group had the highest combined value when compared to 170 other groups and the treatment group 1 had the lowest combined value among the other groups. 171 Both treatment groups did not have any differences in combined value means the administration of 172 banana and banana batu banana resistant starch had the same effect.

173 174 **4. Discussion**

167

175The above results indicate that resistant starch in *batu* and *kepok* banana flours may have176protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit177inflammation due to the induction of AOM and DSS compounds where the level of inflammation is178the same as the groups that are not induced by AOM and DSS compounds.

179 The results of this study support the study conducted by $\frac{1}{1000}$ Hu et al₂ (2016) where the 180 inflammation score decreased in experimental animals that were induced by AOM DSS and given a 181 diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or 182 chemical compounds play an important role in the early stages of colorectal carcinogenesis in which 183 the inflammation may induce chronic immune response resulting in cellular proliferation. (Mariani, 184 et al., 2014). If the immune response fails, cytokines, growth factors and cellular respiration 185 products will continue to proliferate to repair the wound. This can lead to the accumulation of 186 genetic errors and improper proliferation. (Mariani, et al., 2014).

187 The results of this study are reinforced by the results of previous studies where the positive 188 control group that was given only a standard diet had a high inflammatory score and had the 189 number of bacteria from the genus Fusibacterium, Escherichia and Enterococcus which were also 190 associated with CRC in humans (Feng et al., 2015). Administration of AOM which induces DNA 191 damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community 192 which contributes to tumor formation (Zackular et al., 2013). The study also indicated a dynamic 193 change in the microbiota population in the initial response to AOM and DSS before signs of 194 macroscopic tumor formation emerged (Zackular et al., 2013). The crucial role of microbiotic 195 dysbiosis is supported by research where experimental animals that do not experience dysbiosis do 196 not experience inflammation and colon cancer (Vannucci et al., 2008). Hospital administration can 197 increase bacteria associated with RS fermentation such as Parabacteroides, Ruminococcus and 198 Bifidobacterium (Hu et al., 2016). In addition, there is also an increase in bacteria which is not 199 directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing 200 inflammation or regeneration of the colonic mucosa (Wong, et al., 2013; Nava and Stappenbeck, 201 2011).

202SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-203protein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky,2042014). GPR43 is GPR that expressed in intestinal epithelial cells and certain immune cells, but the205expression is lower when individual have CRC and colitis condition (Tang, et al., 2011; Maslowski, et206al., 2009). Previous studies had showed a positive effect of high-fiber diet that can increase207activation of GPR43.(Macia, 2015). Another study showed that RS can increased the activation of208GPR43 expression significantly. Those studies indicates that GPR43 activation may have a role in

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intestinal homeostasis (Hu *et al.*, 2016). Furthemore, acetate and propionate have a significant
 inverse correlation with tumor occurrence by modulate Treg cell and immune function. Those
 indicates an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota
 (Smith, *et al.*, 2013; Fukuda, *et al.*, 2011).

213 High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This may be 214 because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response, 215 inhibiting apoptosis of tumor cells, increasing cell proliferation, regulating the cell cycle, increasing 216 tumor angiogenesis, increasing the expression of metalloproteinases in tumor cells and stimulating 217 the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 218 was reinforced by previous studies where most colon cancers have high COX-2 expression and 219 resulting in tumor angiogenesis, immune system damage and tumor invasion (Brown and DuBois, 220 2005).

221 COX-2 expression in the positive control group was significantly higher than the COX-2 222 expression in the negative control group and the two treatment groups. The results of this study 223 are supported by previous studies that showed the expression of genes associated with 224 inflammation such as COX-2 decreased significantly in the group given RS. The increased expression 225 of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an 226 inflammatory microenvironment that can enhance tissue dysplasia (Hu et al., 2016). RS triggers 227 major changes in colonic gene expression that inhibits inflammatory pathways and suppresses 228 immune responses (Haenen et al., 2013).

229 230

231 Conflict of interest

- 232 The authors declare no conflict of interest.
- 233

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- 347 348

Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES

Component (g)	Mice Group				
Component (g)	К-	K +	P1	P2	
<i>Batu</i> banana flour	-	-	19	-	
Kepok banana flour	-	-	-	18	
Corn starch	46.57	46.57	46.57	46.57	
Protein (casein)	14	14	14	14	
Dectrin	15.5	15.5	5.5	5.5	
Sucrose	10	10	10	10	
Soybean oil	4	4	4	4	
Alpacel (fiber)	5	5	5	5	
Mineral mix	3.5	3.5	3.5	3.5	
Vitamin mix	1	1	1	1	
L-cystine	0.18	0.18	0.18	0.18	
Choline	0.25	0.25	0.25	0.25	

Group	Mean ± Standard Deviation	<i>p</i> -Value
К-	1.32 ± 0.33	
K +	2.48 ± 0.50	
P1	1.56 ± 0.38	0.035 *
P2	1.40 ± 0.47	

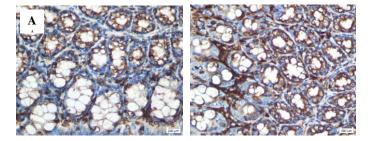
Table 3. The relationship between COX-2 expression with various treatments

a, b, c: different notations in the same column indicate a significant difference

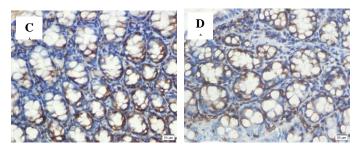
	COX-2				
Variable		Positive	Moderately	Strongly	nyalwa
	n	POSITIVE	positive	positive	p value
Intervention					< 0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM batu treatment	25	12 (48)	7 (28)	6 (24)	
AOM kepok treatment	20	10 (50)	9 (45)	1 (1)	

	Group	COX-2 percentage	COX-2 intensity	Score and COX-2 intensity
		score	,	
	К-	2.52 ± 0.92a	2.48 ± 0.51a	5.00 ± 1.32a
	К +	3.52 ± 0.59b	2.76 ± 0.44b	6.28 ± 0.84b
	P1	1.80 ± 1.32c	2.04 ± 0.74c	3.84 ± 1.86c
	P2	1.40 ± 0.88c	2.35 ± 0.49ac	3.75 ± 1.12c
	p	<0.001 *	<0.001 *	<0.001 *
64	*	: there is a significant diffe	rence	
55	a	a, b, c: different notations ir	the same column indicate a significant dif	ference
56				
67				
369	C			
70		otouulin Eosin stainin *	he calenic call ticque of mice. Autime -	antral group
70 71			he colonic cell tissue of mice. A: negative co roup, magnification 400x; C: treatment grou	
71 72	0			ap 1, magnification
	400x: D: treat	ment group 2, magnificatio	11 400X.	

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- 379 Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group,
- 380 magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification
- 381 400x; D: treatment group 2, magnification 400x.



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31st July 2021

Dear Dr Afifah

ACCEPTANCE LETTER

Food Research, is pleased to inform you that the following manuscript has been accepted for publication in Food Research journal.

Manuscript Title	:	Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate
Authors	:	Pratiwi, S.N., Afifah, D.N., Widyastiti, S.W., Karlowee, V., Anjani, G. and Istiadi, H.

We thank you for your fine contribution to the Food Research journal and encourage you to submit other articles to the Journal.

Yours sincerely,

Professor Dr. Son Radu Chief Editor Food Research



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Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. *Batu* bananas and *kepok* bananas

contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that are induced by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of banana flour on inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group (p = 0.035). The COX-2 score in the treatment group resistant starch of *batu* and *kepok* banana was significantly lower than the positive control group (p<0.001). The COX-2 intensity in both groups was lower than the positive group but not significant (p<0.001). The coX-2 intensity in both the positive control group (p<0.001). The coxie score between the percentage and the intensity of COX-2 expression in the two treatment groups was also had lower than the positive control group (p<0.001). Resistant starch of *batu* and *kepok* banana can inhibit inflammation and suppressed the expression of COX-2.

Keywords: batu banana, Colon cancer, kepok banana, Resistant starch

1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030 (Birt and Phillips, 2014). The incidence of colon cancer would increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk by 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the aetiology of colon cancer (Le Leu *et al.*, 2007) and one study determined that about 80% of colon cancer cases were related to diet (Le Leu *et al.*, 2002). The results indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fibre that is beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan *et al.*, 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al.*, 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al.*, 2002). Butyrate also has chemoprotective

properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al.*, 2002; Wong *et al.*, 2006).

The source of RS is contained in the Indonesian local bananas where the largest component of its starch was found in the pulp. *Batu* banana (*Musa balbisiana* Colla) and *Kepok* banana (*Musa paradisiaca* formatypica) were types of banana that had high resistant starch content of 39.5% and 27.7%, respectively (Musita, 2009). The method used to increase the RS content in banana flour was through autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) (Fuentes-Zaragoza *et al.*, 2010). Current studies had revealed that *kepok* banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase the RS in *batu* banana and *kepok* banana up to 52.95% and 55.8% respectively (Afifah *et al.*, 2018).

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers (Chandrasekharan and Simmons, 2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane production.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS (Hu *et al.*, 2016). Butyrate produced by RS may directly influence the reduction of COX-2 (Jahns *et al.*, 2011) by inhibiting COX-2 transcription elongation (Tong *et al.*, 2005). In addition, a mechanism that might occur was the effect of inflammatory mediators that play a role in the transcriptional activation of COX-2 (Jung *et al.*, 2005; Usami *et al.*, 2008).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas can protect the colon in the cancer induction effect. This study aimed to prove whether the resistant starch of *batu* banana flour and *kepok* banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the Diponegoro University Integrated Laboratory. Experimental animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was done for 11 weeks. This research had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval for Ethical Clearance under number 33/EC/H/FK-UNDIP/IV/2019.

2.1 Experimental animals

This study used male BALB/c mice aged 5 weeks weighing about 20 to 25 g. The animals were acclimatized beforehand for 7 days, then divided into 4 groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 were groups given AOM and DSS induction and feed that had been modified with banana flour *batu* and *kepok* respectively.

Animals were caged in a room that had air conditioning and had a naturally dark setting. They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization. DSS 2% was given the following day for 7 days.

Batu and *kepok* bananas were peeled and washed with water. The bananas were sliced to about 2 mm thin then dried in the sun for 3 days. Dried banana pieces were crushed and sieved with an 80 mesh sieve. Banana flour was treated by autoclaving it at 121°C for 15 mins and were cooled at 4°C for 24 hrs. The pH of banana flour was adjusted with 0.2 M acetate buffer then 2% of pullulanase enzyme was added (v/w of banana flour). The banana flour was incubated at 40°C for 12 hrs at 150 rpm. The reaction was stopped by heating the suspension at 85°C for 5 mins. Banana flour was wrapped in aluminium foil then were autoclaved at 121°C for 15 mins and were cooled at 4°C for 24 hrs.

2.2 Immunohistochemistry

Animals were quickly terminated by dislocation of the cervical and sterilized. The colons were fixed in 10% Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block was serially sectioned using a rotary microtome. The colon sections were stained with hematoxylin-eosin (HE). Immunostaining COX-2 was performed according to the manufacturer's recommendations (Fine Test, Wuhan, China).

The observation of colon tissues was done using a light microscope with 400× magnification. Each colon tissue sample was captured with 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa; the score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima) (Erben *et al.*, 2014).

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 were divided into 5 scores, the score was 0 if the estimated percentage was 0% to 5%; the score was 1 if the estimated percentage was 6% to 25%, the score was 2 if the estimated percentage was 26% to 50%, the score was 3 if the estimated percentage was 51% to 75% and the score was 4 if the estimated percentage was 76% to 100%. The intensity of staining had 3 scores, score 1 if the tissue has a slightly yellow colour, score 2 when it has a brownish-yellow colour and score 3 when it is brown (Wu and Sun, 2015).

The final scores were the sum of the staining intensity score and the cell staining percentage score. The combination of the score would have 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8) (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by using the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) was the most inflamed among other images. Tissue in treatment 1 and treatment 2 was more inflamed than the positive control but the negative control had the least amount of inflammation. Table 2 revealed that each group had a significant difference in inflammatory values (p-Value = 0.035). The positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Furthermore, the inflammation value of the positive control group was significantly different from the negative control group, both treatment groups displayed the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group

did not have a significant difference in both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow colour in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 2 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. Both treatment groups had lower severity with a positive category percentage of 48% and 50%, respectively.

Table 4 reveals that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where the positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences, thus, it could be said that the administration of *batu* and *kepok* banana resistant starch had the same effect on the COX-2.

The results of the combined value between percentages and intensity of COX-2 between groups showed differences. The positive control group had the highest combined value when compared to other groups and treatment group 1 had the lowest combined value among the other groups. Both treatment groups did not have any differences in combined value means the administration of *kepok* banana and *batu* banana resistant starch had the same effect.

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours may have a protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support Hu *et al.* (2016) where the inflammation score decreased in experimental animals that were induced by AOM and DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation (Mariani *et al.*, 2014). If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation (Mariani *et al.*, 2014).

The results of this study are reinforced by previous studies where the positive control group that was given only a standard diet had a high inflammatory score and the number of bacteria from the genus *Fusobacterium, Escherichia* and *Enterococcus* were also associated with CRC in humans (Feng *et al.*, 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumour formation (Zackular *et al.*, 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumour formation emerged (Zackular *et al.*, 2013). The crucial role of microbiota dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer (Vannucci *et al.*, 2008). Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides, Ruminococcus* and *Bifidobacterium* (Hu *et al.*, 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Nava and Stappenbeck, 2011; Wong, *et al.*, 2013).

SCFAs produced by the colonic microbiota can stimulate cell function through activation of Gprotein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 2014). GPR43 is GPR that was expressed at a lower concentration in the intestinal epithelial cells and certain immune cells when an individual has CRC and colitis condition (Maslowski, *et al.*, 2009; Tang *et al.*, 2011). Previous studies had shown that a high-fibre diet has a positive effect that can increase the activation of GPR43 (Macia *et al.*, 2015). Another study showed that RS can increase the activation of GPR43 expression significantly. Those studies indicate that GPR43 activation may have a role in intestinal homeostasis (Hu *et al.*, 2016). Furthermore, acetate and propionate have a significant inverse correlation with tumour occurrence by modulating Treg cell and immune function. Those indicate an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Fukuda, *et al.*, 2011; Smith, *et al.*, 2013).

High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This is because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumour cells, increasing cell proliferation, regulating the cell cycle, increasing tumour angiogenesis, increasing the expression of metalloproteinases in tumour cells and stimulating the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and result in tumour angiogenesis, immune system damage and tumour invasion (Brown and DuBois, 2005). COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia (Hu *et al.*, 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses (Haenen *et al.*, 2013).

Conflict of interest

The authors declare no conflict of interest.

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES

Component (g)	Mice Group				
component (g)	K-	K +	P1	P2	
Batu banana flour	-	-	19	-	
<i>Kepok</i> banana flour	-	-	-	18	
Corn starch	46.57	46.57	46.57	46.57	
Protein (casein)	14	14	14	14	
Dectrin	15.5	15.5	5.5	5.5	
Sucrose	10	10	10	10	
Soybean oil	4	4	4	4	
Alpacel (fiber)	5	5	5	5	
Mineral mix	3.5	3.5	3.5	3.5	
Vitamin mix	1	1	1	1	
L-cystine	0.18	0.18	0.18	0.18	
Choline	0.25	0.25	0.25	0.25	

Table 1. Composition of experimental animal feed g/100 g

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Table 2. Inflammation	after intervention

Group	Mean ± Standard Deviation	<i>p</i> -Value		
К-	1.32 ± 0.33			
К +	2.48 ± 0.50			
P1	1.56 ± 0.38	0.035 *		
P2	1.40 ± 0.47			
*Statistically significant difference (n<0.0E)				

*Statistically significant difference (p<0.05)

a, b, c: different notations in the same column indicate a significant difference

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Table 3. The relationship between COX-2 expression with various treatments

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	COX-2				
Variable		Desitive	Moderately	Strongly	nyalua
	n Positive	POSITIVE	positive	positive	p value
Intervention					< 0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM batu treatment	25	12 (48)	7 (28)	6 (24)	
AOM kepok treatment	20	10 (50)	9 (45)	1 (1)	

Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage	COX-2 intensity	Score and COX-2 intensity
	score		
K-	2.52 ± 0.92a	2.48 ± 0.51a	5.00 ± 1.32a
K +	3.52 ± 0.59b	2.76 ± 0.44b	6.28 ± 0.84b
P1	1.80 ± 1.32c	2.04 ± 0.74c	3.84 ± 1.86c
P2	1.40 ± 0.88c	2.35 ± 0.49ac	3.75 ± 1.12c
p	<0.001 *	<0.001 *	<0.001 *
	10		

*Statistically significant difference

a, b, c: different notations in the same column indicate a significant difference

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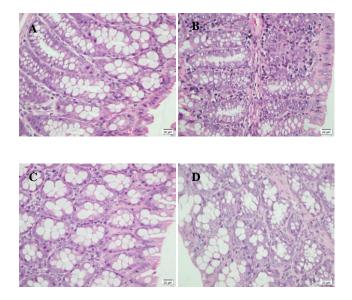


Figure 1. Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

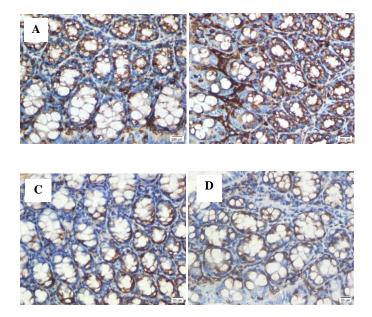


Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.



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Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. *Batu* bananas and *kepok* bananas

contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that are induced by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of banana flour on inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group (p = 0.035). The COX-2 score in the treatment group resistant starch of *batu* and *kepok* banana was significantly lower than the positive control group (p<0.001). The COX-2 intensity in both groups was lower than the positive group but not significant (p<0.001). The coX-2 intensity in both the positive control group (p<0.001). The coxie score between the percentage and the intensity of COX-2 expression in the two treatment groups was also had lower than the positive control group (p<0.001). Resistant starch of *batu* and *kepok* banana can inhibit inflammation and suppressed the expression of COX-2.

Keywords: batu banana, Colon cancer, kepok banana, Resistant starch

1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030 (Birt and Phillips, 2014). The incidence of colon cancer would increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk by 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the aetiology of colon cancer (Le Leu *et al.*, 2007) and one study determined that about 80% of colon cancer cases were related to diet (Le Leu *et al.*, 2002). The results indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fibre that is beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan *et al.*, 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al.*, 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al.*, 2002). Butyrate also has chemoprotective

properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al.*, 2002; Wong *et al.*, 2006).

The source of RS is contained in the Indonesian local bananas where the largest component of its starch was found in the pulp. *Batu* banana (*Musa balbisiana* Colla) and *Kepok* banana (*Musa paradisiaca* formatypica) were types of banana that had high resistant starch content of 39.5% and 27.7%, respectively (Musita, 2009; Afifah *et al.*, 2020). The method used to increase the RS content in banana flour was through autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) (Fuentes-Zaragoza *et al.*, 2010; Ratnasari *et al.*, 2018; Afifah *et al.*, 2018). Current studies had revealed that *kepok* banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase the RS in *batu* banana and *kepok* banana up to 52.95% and 55.8% respectively (Afifah *et al.*, 2021a).

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers (Chandrasekharan and Simmons, 2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane production.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS (Hu *et al.*, 2016). Butyrate produced by RS may directly influence the reduction of COX-2 (Jahns *et al.*, 2011; Afifah *et al.*, 2021b) by inhibiting COX-2 transcription elongation (Tong *et al.*, 2005). In addition, a mechanism that might occur was the effect of inflammatory mediators that play a role in the transcriptional activation of COX-2 (Jung *et al.*, 2005; Usami *et al.*, 2008).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas can protect the colon in the cancer induction effect. This study aimed to prove whether the resistant starch of *batu* banana flour and *kepok* banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the Diponegoro University Integrated Laboratory. Experimental animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was done for 11 weeks. This research had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval for Ethical Clearance under number 33/EC/H/FK-UNDIP/IV/2019.

2.1 Experimental animals

This study used male BALB/c mice aged 5 weeks weighing about 20 to 25 g. The animals were acclimatized beforehand for 7 days, then divided into 4 groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 were groups given AOM and DSS induction and feed that had been modified with banana flour *batu* and *kepok* respectively. The composition of feed for each group can be seen within the Table 1.

Animals were caged in a room that had air conditioning and had a naturally dark setting. They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization. DSS 2% was given the following day for 7 days.

Batu and *kepok* bananas were peeled and washed with water. The bananas were sliced to about 2 mm thin then dried in the sun for 3 days. Dried banana pieces were crushed and sieved with an 80 mesh sieve. Banana flour was treated by autoclaving it at 121°C for 15 mins and were cooled at 4°C for 24 hrs. The pH of banana flour was adjusted with 0.2 M acetate buffer then 2% of pullulanase enzyme was added (v/w of banana flour). The banana flour was incubated at 40°C for 12 hrs at 150 rpm. The reaction was stopped by heating the suspension at 85°C for 5 mins. Banana flour was wrapped in aluminium foil then were autoclaved at 121°C for 15 mins and were cooled at 4°C for 24 hrs.

2.2 Immunohistochemistry

Animals were quickly terminated by dislocation of the cervical and sterilized. The colons were fixed in 10% Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block was serially sectioned using a rotary microtome. The colon sections were stained with hematoxylin-eosin (HE). Immunostaining COX-2 was performed according to the manufacturer's recommendations (Fine Test, Wuhan, China).

The observation of colon tissues was done using a light microscope with 400× magnification. Each colon tissue sample was captured with 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score

was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima) (Erben *et al.*, 2014).

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 were divided into 5 scores, the score was 0 if the estimated percentage was 0% to 5%; the score was 1 if the estimated percentage was 6% to 25%, the score was 2 if the estimated percentage was 26% to 50%, the score was 3 if the estimated percentage was 51% to 75% and the score was 4 if the estimated percentage was 76% to 100%. The intensity of staining had 3 scores, score 1 if the tissue has a slightly yellow colour, score 2 when it has a brownish-yellow colour and score 3 when it is brown (Wu and Sun, 2015).

The final scores were the sum of the staining intensity score and the cell staining percentage score. The combination of the score would have 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8) (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by using the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) was the most inflamed among other images. Tissue in treatment 1 and treatment 2 was more inflamed than the positive control but the negative control had the least amount of inflammation. Table 2 revealed that each group had a significant difference in inflammatory values (p-Value = 0.035). The positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Furthermore, the inflammation value of the positive control group was significantly different from the negative control group, both treatment groups displayed the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not have a significant difference in both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow colour in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 3 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. Both treatment groups had lower severity with a positive category percentage of 48% and 50%, respectively.

Table 4 reveals that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where the positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences, thus, it could be said that the administration of *batu* and *kepok* banana resistant starch had the same effect on the COX-2.

The results of the combined value between percentages and intensity of COX-2 between groups showed differences. The positive control group had the highest combined value when compared to other groups and treatment group 1 had the lowest combined value among the other groups. Both treatment groups did not have any differences in combined value means the administration of *kepok* banana and *batu* banana resistant starch had the same effect.

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours may have a protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support Hu *et al.* (2016) where the inflammation score decreased in experimental animals that were induced by AOM and DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation (Mariani *et al.*, 2014). If the

immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation (Mariani *et al.*, 2014).

The results of this study are reinforced by previous studies where the positive control group that was given only a standard diet had a high inflammatory score and the number of bacteria from the genus *Fusobacterium, Escherichia* and *Enterococcus* were also associated with CRC in humans (Feng *et al.*, 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumour formation (Zackular *et al.*, 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumour formation emerged (Zackular *et al.*, 2013). The crucial role of microbiota dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer (Vannucci *et al.*, 2008). Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides, Ruminococcus* and *Bifidobacterium* (Hu *et al.*, 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Nava and Stappenbeck, 2011; Wong, *et al.*, 2013).

SCFAs produced by the colonic microbiota can stimulate cell function through activation of Gprotein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 2014). GPR43 is GPR that was expressed at a lower concentration in the intestinal epithelial cells and certain immune cells when an individual has CRC and colitis condition (Maslowski, *et al.*, 2009; Tang *et al.*, 2011). Previous studies had shown that a high-fibre diet has a positive effect that can increase the activation of GPR43 (Macia *et al.*, 2015). Another study showed that RS can increase the activation of GPR43 expression significantly. Those studies indicate that GPR43 activation may have a role in intestinal homeostasis (Hu *et al.*, 2016). Furthermore, acetate and propionate have a significant inverse correlation with tumour occurrence by modulating Treg cell and immune function. Those indicate an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Fukuda, *et al.*, 2011; Smith, *et al.*, 2013).

High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This is because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumour cells, increasing cell proliferation, regulating the cell cycle, increasing tumour angiogenesis, increasing the expression of metalloproteinases in tumour cells and stimulating the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and result in tumour angiogenesis, immune system damage and tumour invasion (Brown and DuBois, 2005).

COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia (Hu *et al.*, 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses (Haenen *et al.*, 2013).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES

Component (g)		Mice Group			
component (g)	K-	K +	P1	P2	
<i>Batu</i> banana flour	-	-	19	-	
<i>Kepok</i> banana flour	-	-	-	18	
Corn starch	46.57	46.57	46.57	46.57	
Protein (casein)	14	14	14	14	
Dectrin	15.5	15.5	5.5	5.5	
Sucrose	10	10	10	10	
Soybean oil	4	4	4	4	
Alpacel (fiber)	5	5	5	5	
Mineral mix	3.5	3.5	3.5	3.5	
Vitamin mix	1	1	1	1	
L-cystine	0.18	0.18	0.18	0.18	
Choline	0.25	0.25	0.25	0.25	

Table 1. Composition of experimental animal feed g/100 g

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Table 2. Inflammation after intervention
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Group	Mean ± Standard Deviation	<i>p</i> -Value
К-	1.32 ± 0.33ª	
K +	2.48 ± 0.50 ^b	
P1	1.56 ± 0.38°	0.035 *
P2	$1.40 \pm 0.47^{\circ}$	

*Statistically significant difference (p<0.05)

a, b, c: different notations in the same column indicate a significant difference

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	COX-2					
Variable		Positive	Moderately	Strongly		
	n Po	Positive	positive	positive	<i>p</i> value	
Intervention					<0.001	
Control-negative	25	4 (16)	10 (40)	11 (44)		
AOM positive control	25	0 (0)	4 (16)	21 (84)		
AOM batu treatment	25	12 (48)	7 (28)	6 (24)		
AOM kepok treatment	20	10 (50)	9 (45)	1 (1)		

Table 3. The relationship between COX-2 expression with various treatments

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Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage	COX-2 intensity	Score and COX-2 intensity
	score		
К-	2.52 ± 0.92 ^a	2.48 ± 0.51ª	5.00 ± 1.32 ^a
K +	3.52 ± 0.59 ^b	2.76 ± 0.44^{b}	6.28 ± 0.84^{b}
P1	1.80 ± 1.32 ^c	2.04 ± 0.74 ^c	3.84 ± 1.86 ^c
P2	$1.40 \pm 0.88^{\circ}$	2.35 ± 0.49^{ac}	3.75 ± 1.12 ^c
p	<0.001 *	<0.001 *	<0.001 *

*Statistically significant difference

a, b, c: different notations in the same column indicate a significant difference

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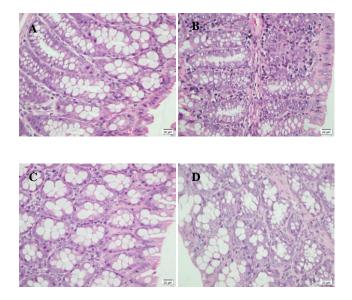


Figure 1. Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

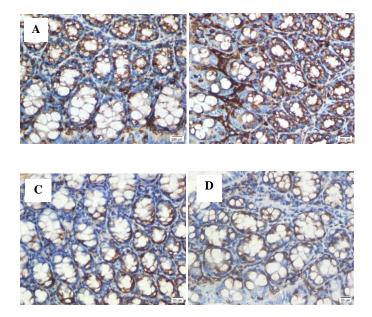


Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.



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Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas contain resistant starch which can inhibit inflammation and COX-2 in mice colon that are induced by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of banana flour on inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group (p = 0.035). The COX-2 score in the treatment group resistant starch of batu and kepok banana was significantly lower than the positive control group (p<0.001). The COX-2 intensity in both groups was lower than the positive group but not significant (p < 0.001). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups was also had lower than the positive control group (p < 0.001). Resistant starch of *batu* and kepok banana can inhibit inflammation and suppressed the expression of COX-2.

1. Introduction

Colon cancer was the third leading cause of cancerrelated death with 130,000 cases every year and was expected to increase by 60% in 2030 (Birt and Phillips, 2014). The incidence of colon cancer would increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk by 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the aetiology of colon cancer (Le Leu *et al.*, 2007) and one study determined that about 80% of colon cancer cases were related to diet (Le Leu *et al.*, 2002). The results indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fibre that is beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan *et al.*, 2009; Purwanti and Suhartono, will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al.*, 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al.*, 2002). Butyrate also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al.*, 2002; Wong *et al.*, 2006). The source of RS is contained in the Indonesian local

2014). In the human body, RS will not be digested but

bananas where the largest component of its starch was found in the pulp. *Batu* banana (*Musa balbisiana* Colla) and *Kepok* banana (*Musa paradisiaca* formatypica) were types of banana that had high resistant starch content of 39.5% and 27.7%, respectively (Musita, 2009; Afifah *et al.*, 2020). The method used to increase the RS content in banana flour was through autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) (Fuentes-Zaragoza *et al.*, 2010; Ratnasari *et al.*, 2018; Afifah *et al.*, 2018). Current studies had revealed that *kepok* banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase the RS in *batu* banana and *kepok* banana up to 52.95% and 55.8% respectively (Afifah *et al.*, 2021a).

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers (Chandrasekharan and Simmons, 2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane production.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS (Hu *et al.*, 2016). Butyrate produced by RS may directly influence the reduction of COX-2 (Jahns *et al.*, 2011; Afifah *et al.*, 2021b) by inhibiting COX-2 transcription elongation (Tong *et al.*, 2005). In addition, a mechanism that might occur was the effect of inflammatory mediators that play a role in the transcriptional activation of COX-2 (Jung *et al.*, 2005; Usami *et al.*, 2008).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas can protect the colon in the cancer induction effect. This study aimed to prove whether the resistant starch of *batu* banana flour and *kepok* banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the Diponegoro University Integrated Laboratory. Experimental animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was done for 11 weeks. This research had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval for Ethical Clearance under number 33/ EC/H/FK-UNDIP/IV/2019.

2.1 Experimental animals

This study used male BALB/c mice aged 5 weeks weighing about 20 to 25 g. The animals were acclimatized beforehand for 7 days, then divided into 4

groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C-will be given AIN-93 feed without induction and any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 were groups given AOM and DSS induction and feed that had been modified with banana flour *batu* and *kepok* respectively. The composition of feed for each group can be seen within the Table 1.

Table 1. Composition of experimental animal feed g/100 g

Common ant (a)		Mice	Group	
Component (g)	K-	K +	P1	P2
Batu banana flour	-	-	19	-
<i>Kepok</i> banana flour	-	-	-	18
Corn starch	46.57	46.57	46.57	46.57
Protein (casein)	14	14	14	14
Dectrin	15.5	15.5	5.5	5.5
Sucrose	10	10	10	10
Soybean oil	4	4	4	4
Alpacel (fiber)	5	5	5	5
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1
L-cystine	0.18	0.18	0.18	0.18
Choline	0.25	0.25	0.25	0.25

Animals were caged in a room that had air conditioning and had a naturally dark setting. They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization. DSS 2% was given the following day for 7 days.

Batu and *kepok* bananas were peeled and washed with water. The bananas were sliced to about 2 mm thin then dried in the sun for 3 days. Dried banana pieces were crushed and sieved with an 80 mesh sieve. Banana flour was treated by autoclaving it at 121°C for 15 mins and were cooled at 4°C for 24 hrs. The pH of banana flour was adjusted with 0.2 M acetate buffer then 2% of pullulanase enzyme was added (v/w of banana flour). The banana flour was incubated at 40°C for 12 hrs at 150 rpm. The reaction was stopped by heating the suspension at 85°C for 5 mins. Banana flour was wrapped in aluminium foil then were autoclaved at 121°C for 15 mins and were cooled at 4°C for 24 hrs.

2.2 Immunohistochemistry

Animals were quickly terminated by dislocation of the cervical and sterilized. The colons were fixed in 10% Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block was serially sectioned using a rotary microtome. The colon sections were stained with hematoxylin-eosin (HE). Immunostaining COX-2 was performed according to the manufacturer's recommendations (Fine Test, Wuhan, China). The observation of colon tissues was done using a light microscope with $400 \times$ magnification. Each colon tissue sample was captured with 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the inflammation infiltrates to the tunica intima) (Erben *et al.*, 2014).

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 were divided into 5 scores, the score was 0 if the estimated percentage was 0% to 5%; the score was 1 if the estimated percentage was 6% to 25%, the score was 2 if the estimated percentage was 26% to 50%, the score was 3 if the estimated percentage was 51% to 75% and the score was 4 if the estimated percentage was 76% to 100%. The intensity of staining had 3 scores, score 1 if the tissue has a slightly yellow colour, score 2 when it has a brownish-yellow colour and score 3 when it is brown (Wu and Sun, 2015).

The final scores were the sum of the staining intensity score and the cell staining percentage score. The combination of the score would have 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination of the combination is 7 up to 8) (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by using the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) was the most inflamed among other images. Tissue in treatment 1 and treatment 2 was more inflamed than the positive control but the negative control had the least amount of inflammation. Table 2 reveals that each group had a significant difference in inflammatory values (p-Value = 0.035). The positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Table 2.	Inflamma	tion afte	r interv	ention

Group	Mean±Standard Deviation	p-Value
K-	1.32±0.33 ^a	
K +	$2.48{\pm}0.50^{b}$	
P1	1.56±0.38°	0.035 *
P2	$1.40{\pm}0.47^{\circ}$	

*Statistically significant difference (p<0.05). Different superscripts within the same column are significantly different.

Furthermore, the inflammation value of the positive control group was significantly different from the negative control group, both treatment groups displayed the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not have a significant difference in both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow colour in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 3 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. Both treatment groups had lower severity with a positive category percentage of 48% and 50%, respectively.

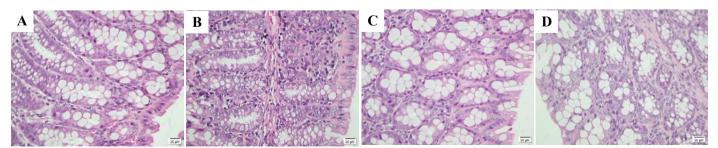


Figure 1. Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification $400\times$; B: positive control group, magnification $400\times$; C: treatment group 1, magnification $400\times$; D: treatment group 2, magnification $400\times$.

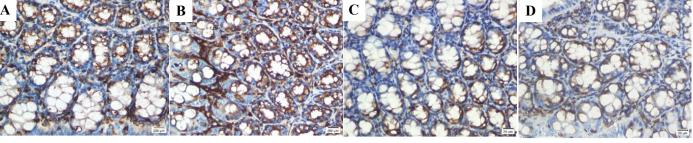


Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification $400\times$; B: positive control group, magnification $400\times$; C: treatment group 1, magnification $400\times$; D: treatment group 2, magnification $400\times$.

Table 3. The relationship between COX-2 expression with various treatments

Variable	COX-2				
variable	n	Positive	Moderately positive	Strongly positive	<i>p</i> -value
Intervention					< 0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM batu treatment	25	12 (48)	7 (28)	6 (24)	
AOM kepok treatment	20	10 (50)	9 (45)	1(1)	

Table 4 reveals that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where the positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences, thus, it could be said that the administration of *batu* and *kepok* banana resistant starch had the same effect on the COX-2.

Table 4. Expression of COX-2 after intervention

Group	COX-2	COX-2 intensity	Score and COX-2
F	percentage score		intensity
К-	$2.52{\pm}0.92^{a}$	$2.48{\pm}0.51^{a}$	$5.00{\pm}1.32^{a}$
K +	$3.52{\pm}0.59^{b}$	2.76 ± 0.44^{b}	$6.28{\pm}0.84^{b}$
P1	$1.80{\pm}1.32^{\circ}$	$2.04{\pm}0.74^{\circ}$	$3.84{\pm}1.86^{\circ}$
P2	$1.40{\pm}0.88^{\circ}$	$2.35{\pm}0.49^{\mathrm{ac}}$	$3.75 \pm 1.12^{\circ}$
р	< 0.001*	<0.001*	< 0.001*

*Statistically significant difference (p<0.05). Different superscripts within the same column are significantly different.

The results of the combined value between percentages and intensity of COX-2 between groups showed differences. The positive control group had the highest combined value when compared to other groups and treatment group 1 had the lowest combined value among the other groups. Both treatment groups did not have any differences in combined value means the administration of *kepok* banana and *batu* banana resistant starch had the same effect.

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours may have a protective effect on AOM and DSS-induced colorectal cancer

initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support Hu *et al.* (2016) where the inflammation score decreased in experimental animals that were induced by AOM and DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation (Mariani *et al.*, 2014). If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation (Mariani *et al.*, 2014).

The results of this study are reinforced by previous studies where the positive control group that was given only a standard diet had a high inflammatory score and the number of bacteria from the genus Fusobacterium, Escherichia and Enterococcus were also associated with CRC in humans (Feng et al., 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumour formation (Zackular et al., 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumour formation emerged (Zackular et al., 2013). The crucial role of microbiota dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer (Vannucci et al., 2008).

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Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides*, *Ruminococcus* and *Bifidobacterium* (Hu *et al.*, 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Nava and Stappenbeck, 2011; Wong, *et al.*, 2013).

SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-protein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 2014). GPR43 is GPR that was expressed at a lower concentration in the intestinal epithelial cells and certain immune cells when an individual has CRC and colitis condition (Maslowski, et al., 2009; Tang et al., 2011). Previous studies had shown that a high-fibre diet has a positive effect that can increase the activation of GPR43 (Macia et al., 2015). Another study showed that RS can increase the activation of GPR43 expression significantly. Those studies indicate that GPR43 activation may have a role in intestinal homeostasis (Hu et al., 2016). Furthermore, acetate and propionate have a significant inverse correlation with tumour occurrence by modulating Treg cell and immune function. Those indicate an antiinflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Fukuda, et al., 2011; Smith, et al., 2013).

High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This is because COX -2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumour cells, increasing cell proliferation, regulating the cell cycle, increasing tumour angiogenesis, increasing the expression of metalloproteinases in tumour cells and stimulating the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and result in tumour angiogenesis, immune system damage and tumour invasion (Brown and DuBois, 2005).

COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia (Hu et al., 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses (Haenen *et al.*, 2013).

Conflict of interest

The authors declare no conflict of interest.

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Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas contain resistant starch which can inhibit inflammation and COX-2 in mice colon that are induced by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of banana flour on inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group (p = 0.035). The COX-2 score in the treatment group resistant starch of *batu* and *kepok* banana was significantly lower than the positive control group (p<0.001). The COX-2 intensity in both groups was lower than the positive group but not significant (p < 0.001). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups was also had lower than the positive control group (p < 0.001). Resistant starch of *batu* and kepok banana can inhibit inflammation and suppressed the expression of COX-2.

1. Introduction

Colon cancer was the third leading cause of cancerrelated death with 130,000 cases every year and was expected to increase by 60% in 2030 (Birt and Phillips, 2014). The incidence of colon cancer would increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk by 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the aetiology of colon cancer (Le Leu *et al.*, 2007) and one study determined that about 80% of colon cancer cases were related to diet (Le Leu *et al.*, 2002). The results indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fibre that is beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan *et al.*, 2009; Purwanti and Suhartono, produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al.*, 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al.*, 2002). Butyrate also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al.*, 2002; Wong *et al.*, 2006). The source of RS is contained in the Indonesian local bananas where the largest component of its starch was

2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will

bananas where the largest component of its starch was found in the pulp. *Batu* banana (*Musa balbisiana* Colla) and *Kepok* banana (*Musa paradisiaca* formatypica) were types of banana that had high resistant starch content of 39.5% and 27.7%, respectively (Musita, 2009; Afifah *et al.*, 2020). The method used to increase the RS content in FULL PAPER

banana flour was through autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) (Fuentes-Zaragoza *et al.*, 2010; Ratnasari *et al.*, 2018; Afifah *et al.*, 2018). Current studies had revealed that *kepok* banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase the RS in *batu* banana and *kepok* banana up to 52.95% and 55.8% respectively (Afifah *et al.*, 2021a).

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers (Chandrasekharan and Simmons, 2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane production.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS (Hu *et al.*, 2016). Butyrate produced by RS may directly influence the reduction of COX-2 (Jahns *et al.*, 2011; Afifah *et al.*, 2021b) by inhibiting COX-2 transcription elongation (Tong *et al.*, 2005). In addition, a mechanism that might occur was the effect of inflammatory mediators that play a role in the transcriptional activation of COX-2 (Jung *et al.*, 2005; Usami *et al.*, 2008).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas can protect the colon in the cancer induction effect. This study aimed to prove whether the resistant starch of *batu* banana flour and *kepok* banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the Diponegoro University Integrated Laboratory. Experimental animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was done for 11 weeks. This research had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval for Ethical Clearance under number 33/ EC/H/FK-UNDIP/IV/2019.

2.1 Experimental animals

This study used male BALB/c mice aged 5 weeks weighing about 20 to 25 g. The animals were acclimatized beforehand for 7 days, then divided into 4

groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C-will be given AIN-93 feed without induction and any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 were groups given AOM and DSS induction and feed that had been modified with banana flour *batu* and *kepok* respectively. The composition of feed for each group can be seen within the Table 1.

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Table 1.	Composition	of experime	ental animal	feed g/100 g

Component (a)	Mice Group				
Component (g)		<u> </u>	<u> </u>	2	
Batu banana flour		-	19		
<i>Kepok</i> banana flour	-	-	-	18	
Corn starch	46.57	46.57	46.57	46.57	
Protein (casein)	14	14	14	14	
Dectrin	15.5	15.5	5.5	5.5	
Sucrose	10	10	10	10	
Soybean oil	4	4	4	4	
Alpacel (fiber)	5	5	5	5	
Mineral mix	3.5	3.5	3.5	3.5	
Vitamin mix	1	1	1	1	
L-cystine	0.18	0.18	0.18	0.18	
Choline	0.25	0.25	0.25	0.25	

Animals were caged in a room that had air conditioning and had a naturally dark setting. They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization. DSS 2% was given the following day for 7 days.

Batu and *kepok* bananas were peeled and washed with water. The bananas were sliced to about 2 mm thin then dried in the sun for 3 days. Dried banana pieces were crushed and sieved with an 80 mesh sieve. Banana flour was treated by autoclaving it at 121°C for 15 mins and were cooled at 4°C for 24 hrs. The pH of banana flour was adjusted with 0.2 M acetate buffer then 2% of pullulanase enzyme was added (v/w of banana flour). The banana flour was incubated at 40°C for 12 hrs at 150 rpm. The reaction was stopped by heating the suspension at 85°C for 5 mins. Banana flour was wrapped in aluminium foil then were autoclaved at 121°C for 15 mins and were cooled at 4°C for 24 hrs.

2.2 Immunohistochemistry

Animals were quickly terminated by dislocation of the cervical and sterilized. The colons were fixed in 10% Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block was serially sectioned using a rotary microtome. The colon sections were stained with hematoxylin-eosin (HE). Immunostaining COX-2 was performed according to the manufacturer's recommendations (Fine Test, Wuhan, China). The observation of colon tissues was done using a light microscope with $400 \times$ magnification. Each colon tissue sample was captured with 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the inflammation infiltrates to the tunica intima) (Erben *et al.*, 2014).

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 were divided into 5 scores, the score was 0 if the estimated percentage was 0% to 5%; the score was 1 if the estimated percentage was 6% to 25%, the score was 2 if the estimated percentage was 26% to 50%, the score was 3 if the estimated percentage was 51% to 75% and the score was 4 if the estimated percentage was 76% to 100%. The intensity of staining had 3 scores, score 1 if the tissue has a slightly yellow colour, score 2 when it has a brownish-yellow colour and score 3 when it is brown (Wu and Sun, 2015).

The final scores were the sum of the staining intensity score and the cell staining percentage score. The combination of the score would have 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination of the combination is 7 up to 8) (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by using the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) was the most inflamed among other images. Tissue in treatment 1 and treatment 2 was more inflamed than the positive control but the negative control had the least amount of inflammation. Table 2 reveals that each group had a significant difference in inflammatory values (p-Value = 0.035). The positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Table 2. Inflammation after intervent	ion
---------------------------------------	-----

Group	Mean±Standard Deviation	p-Value
= K-	$1.32{\pm}0.33^{a}$	
= +	$2.48{\pm}0.50^{b}$	
21	$1.56 \pm 0.38^{\circ}$	0.035 *
29 =	$1.40{\pm}0.47^{c}$	

*Statistically significant difference (p<0.05). Different superscripts within the same column are significantly different.

Furthermore, the inflammation value of the positive control group was significantly different from the negative control group, both treatment groups displayed the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not have a significant difference in both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow colour in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 3 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. Both treatment groups had lower severity with a positive category percentage of 48% and 50%, respectively.

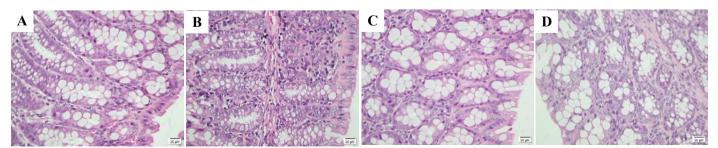


Figure 1. Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification $400\times$; B: positive control group, magnification $400\times$; C: treatment group 1, magnification $400\times$; D: treatment group 2, magnification $400\times$.

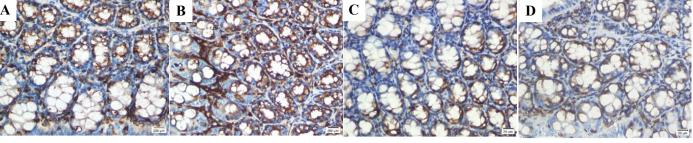


Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification $400\times$; B: positive control group, magnification $400\times$; C: treatment group 1, magnification $400\times$; D: treatment group 2, magnification $400\times$.

Table 3. The relationship between COX-2 expression with various treatments

Variable			COX-2		
variable	n	Positive	Moderately positive	Strongly positive	<i>p</i> -value
Intervention					< 0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM batu treatment	25	12 (48)	7 (28)	6 (24)	
AOM kepok treatment	20	10 (50)	9 (45)	1(1)	

Table 4 reveals that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where the positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences, thus, it could be said that the administration of *batu* and *kepok* banana resistant starch had the same effect on the COX-2.

Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage score	COX-2 intensity	Score and COX-2 intensity
= K-	2.52±0.92 ^a	2.48±0.51 ^a	5.00±1.32ª
= (+	$3.52{\pm}0.59^{b}$	2.76 ± 0.44^{b}	$6.28{\pm}0.84^{b}$
<u> </u>	$1.80{\pm}1.32^{\circ}$	$2.04{\pm}0.74^{\circ}$	$3.84{\pm}1.86^{\circ}$
= 22	$1.40{\pm}0.88^{\circ}$	$2.35{\pm}0.49^{\mathrm{ac}}$	$3.75 \pm 1.12^{\circ}$
p	< 0.001*	<0.001*	<0.001*

*Statistically significant difference (p < 0.05). Different superscripts within the same column are significantly different.

The results of the combined value between percentages and intensity of COX-2 between groups showed differences. The positive control group had the highest combined value when compared to other groups and treatment group 1 had the lowest combined value among the other groups. Both treatment groups did not have any differences in combined value means the administration of *kepok* banana and *batu* banana resistant starch had the same effect.

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours may have a protective effect on AOM and DSS-induced colorectal cancer

initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support Hu *et al.* (2016) where the inflammation score decreased in experimental animals that were induced by AOM and DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation (Mariani *et al.*, 2014). If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation (Mariani *et al.*, 2014).

The results of this study are reinforced by previous studies where the positive control group that was given only a standard diet had a high inflammatory score and the number of bacteria from the genus Fusobacterium, Escherichia and Enterococcus were also associated with CRC in humans (Feng et al., 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumour formation (Zackular et al., 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumour formation emerged (Zackular et al., 2013). The crucial role of microbiota dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer (Vannucci et al., 2008).

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Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides*, *Ruminococcus* and *Bifidobacterium* (Hu *et al.*, 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Nava and Stappenbeck, 2011; Wong, *et al.*, 2013).

SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-protein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 2014). GPR43 is GPR that was expressed at a lower concentration in the intestinal epithelial cells and certain immune cells when an individual has CRC and colitis condition (Maslowski, et al., 2009; Tang et al., 2011). Previous studies had shown that a high-fibre diet has a positive effect that can increase the activation of GPR43 (Macia et al., 2015). Another study showed that RS can increase the activation of GPR43 expression significantly. Those studies indicate that GPR43 activation may have a role in intestinal homeostasis (Hu et al., 2016). Furthermore, acetate and propionate have a significant inverse correlation with tumour occurrence by modulating Treg cell and immune function. Those indicate an antiinflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Fukuda, et al., 2011; Smith, et al., 2013).

High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This is because COX -2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumour cells, increasing cell proliferation, regulating the cell cycle, increasing tumour angiogenesis, increasing the expression of metalloproteinases in tumour cells and stimulating the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and result in tumour angiogenesis, immune system damage and tumour invasion (Brown and DuBois, 2005).

COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia (Hu et al., 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses (Haenen *et al.*, 2013).

Conflict of interest

The authors declare no conflict of interest.

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Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas contain resistant starch which can inhibit inflammation and COX-2 in mice colon that are induced by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of banana flour on inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group (p = 0.035). The COX-2 score in the treatment group resistant starch of batu and kepok banana was significantly lower than the positive control group (p<0.001). The COX-2 intensity in both groups was lower than the positive group but not significant (p < 0.001). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups was also had lower than the positive control group (p < 0.001). Resistant starch of *batu* and kepok banana can inhibit inflammation and suppressed the expression of COX-2.

1. Introduction

Colon cancer was the third leading cause of cancerrelated death with 130,000 cases every year and was expected to increase by 60% in 2030 (Birt and Phillips, 2014). The incidence of colon cancer would increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk by 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the aetiology of colon cancer (Le Leu *et al.*, 2007) and one study determined that about 80% of colon cancer cases were related to diet (Le Leu *et al.*, 2002). The results indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fibre that is beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan *et al.*, 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al.*, 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al.*, 2002). Butyrate also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al.*, 2002; Wong *et al.*, 2006).

The source of RS is contained in the Indonesian local bananas where the largest component of its starch was found in the pulp. *Batu* banana (*Musa balbisiana* Colla) and *Kepok* banana (*Musa paradisiaca* formatypica) were types of banana that had high resistant starch content of 39.5% and 27.7%, respectively (Musita, 2009; Afifah *et al.*, 2020). The method used to increase the RS content in

banana flour was through autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) (Fuentes-Zaragoza *et al.*, 2010; Ratnasari *et al.*, 2018; Afifah *et al.*, 2018). Current studies had revealed that *kepok* banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase the RS in *batu* banana and *kepok* banana up to 52.95% and 55.8% respectively (Afifah *et al.*, 2021a).

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers (Chandrasekharan and Simmons, 2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane production.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS (Hu *et al.*, 2016). Butyrate produced by RS may directly influence the reduction of COX-2 (Jahns *et al.*, 2011; Afifah *et al.*, 2021b) by inhibiting COX-2 transcription elongation (Tong *et al.*, 2005). In addition, a mechanism that might occur was the effect of inflammatory mediators that play a role in the transcriptional activation of COX-2 (Jung *et al.*, 2005; Usami *et al.*, 2008).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas can protect the colon in the cancer induction effect. This study aimed to prove whether the resistant starch of *batu* banana flour and *kepok* banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the Diponegoro University Integrated Laboratory. Experimental animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was done for 11 weeks. This research had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval for Ethical Clearance under number 33/ EC/H/FK-UNDIP/IV/2019.

2.1 Experimental animals

This study used male BALB/c mice aged 5 weeks weighing about 20 to 25 g. The animals were acclimatized beforehand for 7 days, then divided into 4

groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C-will be given AIN-93 feed without induction and any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 were groups given AOM and DSS induction and feed that had been modified with banana flour *batu* and *kepok* respectively. The composition of feed for each group can be seen within the Table 1.

Table 1. Composition of experimental animal feed g/100 g

Common ant (a)	Mice Group				
Component (g)	C-	C +	T1	T2	
Batu banana flour	-	-	19	-	
<i>Kepok</i> banana flour	-	-	-	18	
Corn starch	46.57	46.57	46.57	46.57	
Protein (casein)	14	14	14	14	
Dectrin	15.5	15.5	5.5	5.5	
Sucrose	10	10	10	10	
Soybean oil	4	4	4	4	
Alpacel (fiber)	5	5	5	5	
Mineral mix	3.5	3.5	3.5	3.5	
Vitamin mix	1	1	1	1	
L-cystine	0.18	0.18	0.18	0.18	
Choline	0.25	0.25	0.25	0.25	

Animals were caged in a room that had air conditioning and had a naturally dark setting. They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization. DSS 2% was given the following day for 7 days.

Batu and *kepok* bananas were peeled and washed with water. The bananas were sliced to about 2 mm thin then dried in the sun for 3 days. Dried banana pieces were crushed and sieved with an 80 mesh sieve. Banana flour was treated by autoclaving it at 121°C for 15 mins and were cooled at 4°C for 24 hrs. The pH of banana flour was adjusted with 0.2 M acetate buffer then 2% of pullulanase enzyme was added (v/w of banana flour). The banana flour was incubated at 40°C for 12 hrs at 150 rpm. The reaction was stopped by heating the suspension at 85°C for 5 mins. Banana flour was wrapped in aluminium foil then were autoclaved at 121°C for 15 mins and were cooled at 4°C for 24 hrs.

2.2 Immunohistochemistry

Animals were quickly terminated by dislocation of the cervical and sterilized. The colons were fixed in 10% Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block was serially sectioned using a rotary microtome. The colon sections were stained with hematoxylin-eosin (HE). Immunostaining COX-2 was performed according to the manufacturer's recommendations (Fine Test, Wuhan, China). The observation of colon tissues was done using a light microscope with $400 \times$ magnification. Each colon tissue sample was captured with 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the inflammation infiltrates to the tunica intima) (Erben *et al.*, 2014).

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 were divided into 5 scores, the score was 0 if the estimated percentage was 0% to 5%; the score was 1 if the estimated percentage was 6% to 25%, the score was 2 if the estimated percentage was 26% to 50%, the score was 3 if the estimated percentage was 51% to 75% and the score was 4 if the estimated percentage was 76% to 100%. The intensity of staining had 3 scores, score 1 if the tissue has a slightly yellow colour, score 2 when it has a brownish-yellow colour and score 3 when it is brown (Wu and Sun, 2015).

The final scores were the sum of the staining intensity score and the cell staining percentage score. The combination of the score would have 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination of the combination is 7 up to 8) (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by using the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) was the most inflamed among other images. Tissue in treatment 1 and treatment 2 was more inflamed than the positive control but the negative control had the least amount of inflammation. Table 2 reveals that each group had a significant difference in inflammatory values (p-Value = 0.035). The positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Table 2.	Inflamn	nation	after	interventi	on

Group	Mean±Standard Deviation	p-Value
C-	1.32±0.33 ^a	
C+	$2.48{\pm}0.50^{b}$	
T1	$1.56 \pm 0.38^{\circ}$	0.035 *
T2	$1.40{\pm}0.47^{c}$	

*Statistically significant difference (p<0.05). Different superscripts within the same column are significantly different.

Furthermore, the inflammation value of the positive control group was significantly different from the negative control group, both treatment groups displayed the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not have a significant difference in both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow colour in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 3 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. Both treatment groups had lower severity with a positive category percentage of 48% and 50%, respectively.

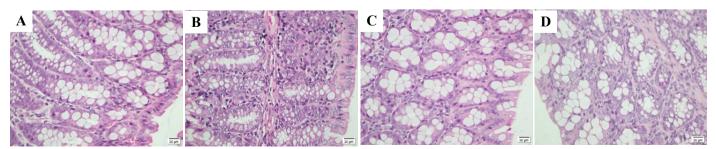


Figure 1. Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification $400\times$; B: positive control group, magnification $400\times$; C: treatment group 1, magnification $400\times$; D: treatment group 2, magnification $400\times$.

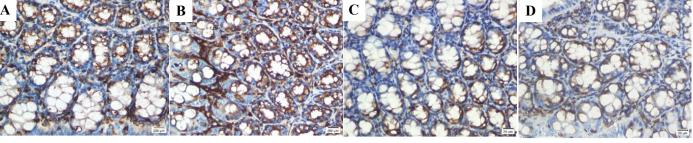


Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification $400\times$; B: positive control group, magnification $400\times$; C: treatment group 1, magnification $400\times$; D: treatment group 2, magnification $400\times$.

Table 3. The relationship between COX-2 expression with various treatments

Variable			COX-2		
variable	n	Positive	Moderately positive	Strongly positive	<i>p</i> -value
Intervention					< 0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM batu treatment	25	12 (48)	7 (28)	6 (24)	
AOM kepok treatment	20	10 (50)	9 (45)	1(1)	

Table 4 reveals that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where the positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences, thus, it could be said that the administration of *batu* and *kepok* banana resistant starch had the same effect on the COX-2.

Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage score	COX-2 intensity	Score and COX-2 intensity
C-	2.52±0.92 ^a	2.48±0.51ª	5.00±1.32 ^a
C+	3.52 ± 0.59^{b}	2.76 ± 0.44^{b}	6.28 ± 0.84^{b}
0			
T1	$1.80 \pm 1.32^{\circ}$	$2.04\pm0.74^{\circ}$	$3.84 \pm 1.86^{\circ}$
T2	$1.40{\pm}0.88^{\circ}$	2.35 ± 0.49^{ac}	$3.75 \pm 1.12^{\circ}$
р	< 0.001*	<0.001*	< 0.001*

*Statistically significant difference (p<0.05). Different superscripts within the same column are significantly different.

The results of the combined value between percentages and intensity of COX-2 between groups showed differences. The positive control group had the highest combined value when compared to other groups and treatment group 1 had the lowest combined value among the other groups. Both treatment groups did not have any differences in combined value means the administration of *kepok* banana and *batu* banana resistant starch had the same effect.

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours may have a protective effect on AOM and DSS-induced colorectal cancer

initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support Hu *et al.* (2016) where the inflammation score decreased in experimental animals that were induced by AOM and DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation (Mariani *et al.*, 2014). If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation (Mariani *et al.*, 2014).

The results of this study are reinforced by previous studies where the positive control group that was given only a standard diet had a high inflammatory score and the number of bacteria from the genus Fusobacterium, Escherichia and Enterococcus were also associated with CRC in humans (Feng et al., 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumour formation (Zackular et al., 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumour formation emerged (Zackular et al., 2013). The crucial role of microbiota dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer (Vannucci et al., 2008).

4

Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides*, *Ruminococcus* and *Bifidobacterium* (Hu *et al.*, 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Nava and Stappenbeck, 2011; Wong, *et al.*, 2013).

SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-protein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 2014). GPR43 is GPR that was expressed at a lower concentration in the intestinal epithelial cells and certain immune cells when an individual has CRC and colitis condition (Maslowski, et al., 2009; Tang et al., 2011). Previous studies had shown that a high-fibre diet has a positive effect that can increase the activation of GPR43 (Macia et al., 2015). Another study showed that RS can increase the activation of GPR43 expression significantly. Those studies indicate that GPR43 activation may have a role in intestinal homeostasis (Hu et al., 2016). Furthermore, acetate and propionate have a significant inverse correlation with tumour occurrence by modulating Treg cell and immune function. Those indicate an antiinflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Fukuda, et al., 2011; Smith, et al., 2013).

High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This is because COX -2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumour cells, increasing cell proliferation, regulating the cell cycle, increasing tumour angiogenesis, increasing the expression of metalloproteinases in tumour cells and stimulating the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and result in tumour angiogenesis, immune system damage and tumour invasion (Brown and DuBois, 2005).

COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia (Hu et al., 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses (Haenen *et al.*, 2013).

Conflict of interest

The authors declare no conflict of interest.

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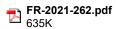
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