Effects of Chitosan and Nitrite on the Properties of Fermented Meat

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Abstract The inhibitory effect of chitosan (0-1%) against some spoilage bacteria in beef was examined. Chitosan (0.5-1%) inhibited Micrococci, Staphylococci, Pseudomonades and Coliforms. This effect increased with increase in chitosan content and incubation time. The effects of combination of three treatments ; chitosan (0.5%), *Lactobacillus plantarum* starter culture and nitrite (100 ppm) on the properties of Gram-negative bacteria, lipid oxidation (TBA value), residual nitrite and color quality in meat patties during fermentation at 30°C were also compared. Treatment of meat with chitosan or *Lactobacillus plantarum* inhibited the growth of Gram-negative bacteria by 1 log cycle, repressed TBA value by 30% and 8% and dissipated residual nitrite by 52% and 60%, respectively. Nitrite treatment inhibited the TBA value and the development of meat color, but had no significant effect on the growth of Gram-negative bacteria. The use of the three treatments together inhibited Gram-negative bacteria by about 2 log cycles, repressed TBA value by 36%, dissipated residual nitrite by 63% and resulted in better color of fermented meat. These results indicated that chitosan could provide meat's protection against spoilage bacteria and could repress its TBA value particularly when combined with nitrite and *Lactobacillus plantarum* fermentation.

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Chitosan, a waste product of the shellfish industry, has been shown to be non toxic²⁾, safe¹¹⁾ and to increase IgM production in human hybridoma cells²¹⁾. Chitosan already has a number of uses in the food industry. It has been patented as a lipid binding food additive⁹⁾, demonstrated its emulsion properties and dye binding capacity¹⁷⁻¹⁹⁾. In the preservation of fruits, it has been used as a coating and antifungal agent, resulting in increased quality and storability of fresh strawberries¹⁰⁾. It has also been used for the reduction of titratable acidity and color index of carrot and apple juices¹²⁾. The antimicrobial activity of chitosan has been described by PAPINEAU *et al.*²⁴⁾ and SUDARSHAN *et*

*al.*²⁸⁾. The utilization of chitosan for the development of the preservative qualities of meat has been presented by DARMADJI *et al.*⁷⁾. Also, chitosan has shown an antimicrobial activity against meat spoilage bacteria and repressed its TBA value. For the development of the preservative quality of traditional fermented meat products, *Lactobacillus plantarum* have been used as a starter culture. Its addition inhibits the growth of some spoilage bacteria, represses TBA value, dissipates residual nitrite and develops the preservative qualities of meat products⁶⁾.

Since fermented meat products generally contain nitrite as a curing agent for color and flavor development as well as preservative

effect, this investigation describes a study on the inhibitory effect of chitosan against some spoilage bacteria in meat, and a study on the effects of chitosan and nitrite on the properties of meat fermented with *Lactobacillus plantarum*.

Materials and Methods

Beef samples were purchased from the local market on the day of preparation. Meat was cut and minced through a 4 mm plate diameter with a meat grinder. The starter culture used was *Lactobacillus plantarum* IAM 1216. Chitosan was obtained from Katokichi Co., Ltd. Japan.

The inhibitory effect of chitosan against some spoilage bacteria in meat: Each 10 g sample of minced meat was mixed with chitosan (0, 0.2, 0.5, 1%) in three replications. Each sample was then wrapped with saran wrap (Asahi Kasei Co., Ltd.) and incubated at 30°C for 12 or 24 h. For the measurement of the inhibitory effect of chitosan, 10 g of each sample was dissolved in 90 ml of sterilized phosphate buffered saline and homogenized with Physcotron (Niton Co., Ltd.). One ml of homogenates was taken and diluted stepwise with 9 ml of sterilized phosphate buffered saline. Appropriate diluents were placed on the following media; Plate Count Agar (PCA) (Difco Co., Ltd.) supplemented with 10% NaCl for Micrococci²⁷⁾; Vogel Johnson Agar (Eiken Ltd.) for Staphylococci; Desoxycholate agar (Nissui Co., Ltd.) for Coliforms⁴⁾; and Bacto OF Basal medium (oxidation/fermentation medium; Difco Co., Ltd.) supplemented with 7.5 mg of Nalidixic acid and 30 mg of Novobiocin in 10 ml of sterile distilled water for Pseudomonades.

OF Basal medium surface plates were incubated at 25°C for 48 h. PCA pour plates were incubated 30°C for 48 h. Vogel Johnson surface plates were incubated at 35°C for 48 h. For Coliforms, pour plates were made of each dilution using 1 ml in Desoxycholate agar. After the pour plates solidified, they were cov-

ered with an additional 5 ml of Desoxycholate agar, incubated at 35°C for 24 h and the red colonies were counted⁴⁾. The inhibitory effect was calculated, as²⁹⁾;

Inhibitory effect:

$$\left(1 - \frac{\text{CFU of prepared sample}}{\text{CFU of control sample}}\right) \times 100\%$$

Effects of chitosan and nitrite on the quality of fermented meat: Minced meat was divided into 8 parts of each 10 g by combination with or without 0.5% chitosan, 100 ppm nitrite and 1% (v/w) *Lactobacillus plantarum* (in YPG medium²³⁾; incubated at 30°C for 16 h, 10⁶ CFU). The samples with and without nitrite were described as cured meat and intact meat respectively. Each 10 g sample was placed in a sterilized plastic petri-dish (35×10 mm; Corning, Iwaki Co., Ltd.). Samples were incubated at 30°C for 12 or 24 h, and were used for microbial analysis, pH, TBA and residual nitrite determination.

Microbiological analysis: Ten g of each sample was dissolved in 40 ml of sterilized distilled water and homogenized with Physcotron. pH was measured with Twin compact pH meter B-112 (Horiba Co., Ltd.). The solution was then made up to 90 ml with sterilized distilled water and homogenized. Gram negative bacteria and *Lactobacillus* were analyzed using Crystal Violet Tetrazolium (CVT) agar (Eiken Ltd.)²⁶⁾ and MRS agar (Oxoid Ltd.) in triplicates. The MRS pour and the CVT surface plates were incubated at 30°C for 48 h.

Chemical analysis: Nitrite was determined by modified AOAC³⁰⁾ methods as follows. The homogenate sample for the microbial analysis was held for approximately 30 minutes for extraction. Ten ml of the homogenized sample was pipetted into a tube, heated in steam bath at 80°C for 2 h with occasional shaking, cooled to room temperature and filtered with Toyo filter paper No. 2. One ml of the filtrate was taken and diluted with 9 ml of distilled water for residual nitrite determination by adding of 0.5 ml sulfanilamide reagent and NED (Naph-

tylethylenediamine) reagent and expressed as ppm of NO₂. The other part of the homogenized sample was used for the determination of TBA (index as lipid oxidation)^{1,16)} and expressed as mg malonaldehyde/kg meat.

Color; Each 40 g of meat samples was placed into plastic petri-dish (60×15 mm; Corning, Iwaki Co., Ltd.), pressed and wrapped with saran wrap before closing with petri-cup. The color of the meat surface was measured with Reflectance Color Meter (CM-1000, Minolta Co., Ltd.) and expressed as L* (lightness), a* (redness) and b* (yellowness) values.

Data analysis: Samples were indicated by the symbols, M, MC, ML, MCL, MCLN, MCN, MNL and MN. The symbols M, C, N and L represents meat, chitosan, nitrite and *Lactobacillus plantarum*, respectively. All data were evaluated statistically using Student-*t* method.

Results and discussions

The inhibitory effect of chitosan against some spoilage bacteria in meat during incuba-

tion at 30°C for 24 h is shown in Table 1. Micrococci, Staphylococci, Pseudomonades and Coliforms were inhibited by chitosan and the inhibition was greater with the increasing incubation time and chitosan concentration. The presence of chitosan inhibited slightly the growth of *Lactobacillus* as shown in Table 2. The growth of Gram-negative bacteria was also inhibited by the presence of chitosan as shown in Table 4. The mode of inhibition of chitosan on the growth of some bacteria might be due to the polycationic nature of chitosan interferes with negatively charged of macromolecules at the cell surface and interaction of chitosan with membranes or cell wall components resulting in increasing permeability of the membranes and leakage of cell materials from tissue³¹⁾. This condition enhances the flow of nutrients and may can facilitate pathogenic infection to the bacterial cell, which causes the inhibition of their growth. In addition, biosorption activities of chitosan²⁰⁾ may remove some nutrient from the cell. The

Table 1. Inhibitory effect of chitosan against some spoilage bacteria in meat during incubation at 30°C

Incubation time (h)		Chitosan (%)					
		0.0		0.2		0.5	
		log CFU (/g)		log CFU (/g)	Inhibition (%)	log CFU (/g)	Inhibition (%)
Pseudo-monades	0	3.65		3.61	8.8	3.60	11.1
	12	5.32		5.17	28.6	4.94	58.0
	24	5.66		5.46	37.1	4.95	80.6
Staphylococci	0	3.17		3.08	20.0	3.00	33.3
	12	5.00		4.57	63.9	4.15	86.0
	24	6.04		5.78	45.5	5.00	90.0
Coliform	0	3.39		3.32	16.0	3.30	20.0
	12	5.43		5.40	7.4	5.36	14.8
	24	8.84		8.66	34.2	8.40	64.2
Micrococci	0	4.23		4.23	0.0	4.20	5.8
	12	6.08		5.08	90.0	4.60	96.6
	24	7.46		6.20	94.5	5.74	98.1

Inhibition : $(1 - \frac{\text{CFU of prepared sample}}{\text{CFU of control sample}}) \times 100\%$

Values are means from triplicate samples.

Table 2. Effects of chitosan, *Lactobacillus plantarum* and nitrite on the growth of *Lactobacillus* of meat during fermentation at 30°C

Treatment	Log CFU/g of <i>Lactobacillus</i>		
	Fermentation time (h)		
	0	12	24
M	4.5 ^a	5.8 ^a	6.6 ^a
MC	4.3 ^b	5.4 ^b	6.4 ^b
ML	6.5 ^c	7.7 ^c	9.0 ^c
MCL	6.5 ^c	7.5 ^d	8.1 ^d
MCLN	6.5 ^c	7.5 ^d	8.1 ^d
MCN	4.3 ^b	5.3 ^b	6.0 ^e
MLN	6.4 ^c	7.7 ^c	8.9 ^c
MN	4.3 ^b	5.8 ^a	6.6 ^a

The different superscript letter within column are significantly different ($p < 0.05$) for triplicate samples.

Treatment

- M : Meat
 MC : Meat+Chitosan
 ML : Meat+*L. plantarum*
 MCL : Meat+Chitosan+*L. plantarum*
 MCLN: Meat+Chitosan+*L. plantarum*+Nitrite
 MCN : Meat+Chitosan+Nitrite
 MLN : Meat+*L. plantarum*+Nitrite
 MN : Meat+Nitrite
 Chitosan : 0.5%
L. plantarum : 1% (10^6 CFU)
 Nitrite : 100 ppm

binding activity of chitosan toward DNA can inhibit mRNA and protein synthesis and its polycations may also play a role in the activation and inactivation of various enzymes¹⁸. These conditions may cause the imbalance in the bacterial cell system leading to the growth inhibition effect observed here.

Since Gram-negative bacteria are generally spoilage bacteria in meat, their growth depression by chitosan may prolong the shelf-life of meat.

The effect of chitosan on the pH of meat is shown in Table 3. Meat containing chitosan had higher pH than meat with no chitosan. That was due to the ability of chitosan to prevent excessive acid production¹² and also

Table 3. Effects of chitosan, *Lactobacillus plantarum* and nitrite on pH of meat during fermentation at 30°C

Treatment ¹⁾	Fermentation time (h)		
	0	12	24
M	6.0 ^a	5.8 ^a	5.7 ^a
MC	6.2 ^b	5.9 ^b	5.8 ^b
ML	5.8 ^c	5.2 ^c	4.6 ^c
MCL	6.1 ^d	5.6 ^d	5.2 ^d
MCLN	6.2 ^b	5.6 ^d	5.2 ^d
MCN	6.3 ^e	5.9 ^b	5.6 ^c
MLN	5.9 ^c	5.3 ^e	4.6 ^c
MN	6.0 ^a	5.7 ^f	5.7 ^a

The different superscript letter within column are significantly different ($p < 0.05$) for duplicate samples.

¹⁾ Symbols are same as shown in Table 2.

Table 4. Effects of chitosan, *Lactobacillus plantarum* and nitrite on the growth of Gram-negative bacteria in meat during fermentation at 30°C

Treatment ¹⁾	Log CFU/g of Gram-negative bact.		
	Fermentation time (h)		
	0	12	24
M	4.6 ^a	6.0 ^a	8.1 ^a
MC	4.5 ^a	5.8 ^{bc}	6.4 ^b
ML	4.7 ^a	5.7 ^{bd}	6.9 ^c
MCL	4.6 ^a	5.6 ^d	6.1 ^d
MCLN	4.6 ^a	5.6 ^d	6.1 ^d
MCN	4.5 ^a	5.8 ^{bc}	6.4 ^b
MLN	4.6 ^a	5.9 ^{ac}	7.0 ^c
MN	4.6 ^a	6.0 ^a	8.1 ^a

The different superscript letter within column are significantly different ($p < 0.05$) for triplicate samples.

¹⁾ Symbols are same as shown in Table 2.

due to by slight inhibition of *Lactobacillus plantarum* accounting for the mild decrease in pH value of meat treated with chitosan and *Lactobacillus plantarum*. This observation is importance in the manufacture of intermediate fermented meats since combination of chitosan and *Lactobacillus plantarum* resulted in the inhibition of meat spoilage microorganisms and

prevented the pH decrease in meat.

The rate of lipid oxidation in meat was reduced by addition of chitosan and *Lactobacillus plantarum* as indicated by repression in TBA value of meat during fermentation at 30°C as shown in Table 5. The TBA value of meat containing *Lactobacillus plantarum* or chitosan decreased by 8% and 30%, respectively. A combined treatment with of chitosan and *Lactobacillus plantarum* repressed the TBA value by 36%. The TBA values of cured meat remained low throughout the experimental period. It is suggested that there could have been an interference reaction between TBA reactive substances and nitrite. However, the combined treatment with *Lactobacillus plantarum* and chitosan in cured meat resulted in lower in TBA value than that of control. Have been reported that *Lactobacillus* strains demonstrated the catalase activities which have ability to decompose hydrogen peroxide when grown in the meat³⁾. So, the inhibition of TBA value in meat prepared with *Lactobacillus plantarum* may be due to the production of catalase like substances which could retard oxidation, resulting in inhibition of lipid oxida-

tion.

The inhibition of TBA value of meat in the presence of chitosan might be due to the lipid binding activity of chitosan⁹⁾ which resulted in the prevention of lipids from oxidation. The amino group of chitosan can also react with volatile malonaldehyde to form a stable fluorophore²⁰⁾. However, in our experiment, chitosan was mixed with meat from at the beginning of experiment and as seen in Table 5. TBA value of sample treated with and without chitosan were the same at 0 h. This result suggests that chitosan does not interfere with TBA reactive substances. It has been reported shrimp meat contain natural antioxidant²⁵⁾ and may contain chitosan. This implies that chitosan has antioxidative properties. Whatever the exact mechanisms of the inhibitory effect on TBA value of meat is unknown, this results indicates that *Lactobacillus plantarum* and chitosan when added to meat could inhibit TBA values and are useful for the depression of lipid oxidation in fermented meat especially when applied in combined form.

Table 6 shows that residual nitrite in cured meat decreased immediately by about 20% and continued to decrease during fermentation at 30°C. When nitrite was added to meat for

Table 5. Effects of chitosan, *Lactobacillus plantarum* and nitrite on TBA value of meat during fermentation at 30°C

Treatment ¹⁾	mg Malonaldehyde/kg meat		
	Fermentation time (h)		
	0	12	24
M	1.42 ^a	2.78 ^a	4.33 ^a
MC	1.45 ^a	1.83 ^b	3.01 ^b
ML	1.46 ^a	2.45 ^c	4.00 ^c
MCL	1.46 ^a	1.75 ^d	2.80 ^d
MCLN	0.83 ^b	0.74 ^e	1.44 ^e
MCN	0.82 ^b	1.09 ^f	1.52 ^{f,g}
MLN	0.82 ^b	1.03 ^g	1.48 ^f
MN	0.92 ^b	1.09 ^f	1.55 ^g

The different superscript letter within column are significantly different ($p < 0.05$) for triplicate samples.

¹⁾ Symbols are same as shown in Table 2.

Table 6. Effects of chitosan, *Lactobacillus plantarum* on residual nitrite of meat containing nitrite during fermentation at 30°C

Treatment ¹⁾	ppm of residual nitrite		
	Fermentation time (h)		
	0	12	24
MN	81.0 ^a	56.6 ^a	44.6 ^a
MCN	77.3 ^b	52.3 ^b	21.5 ^b
MLN	76.0 ^c	27.6 ^c	17.8 ^c
MCLN	76.2 ^c	27.1 ^d	15.7 ^d

The different superscript letter within column are significantly different ($p < 0.05$) for triplicate samples.

¹⁾ Symbols are same as shown in Table 2.

curing, less than 63% of residual nitrite could be detected after addition immediately^{6,22)} and the nitrite in cured meat was converted to nitrate, nitrosomyoglobin and gaseous nitrogen compounds²²⁾. It was observed that, the residual nitrite of cured meat treated with chitosan and *Lactobacillus plantarum* decreased more than that of control. The lower pH by the action of *Lactobacillus plantarum* support the degradation of nitrite to nitric oxide reactive substances⁸⁾. Nitrite could also have reacted with amino or glucosamine of chitosan

to give unstable 2, 5-anhydroderivative⁵⁾. These action may have caused a decrease in the residual nitrite.

The effects of chitosan and *Lactobacillus plantarum* on the color of beef patties during fermentation at 30°C for 24 h are shown in Table 7. The L* value of both intact and cured meat samples containing chitosan was found to decrease. This might have been caused by the increase of water binding capacity¹⁸⁾, resulting in increased transparency and low lighthness¹⁵⁾. In fermented meat, the

Table 7. Effects of chitosan, *Lactobacillus plantarum* and nitrite on color of meat during fermentation at 30°C

Fermentation time (h)	Treatment ¹⁾	L*	a*	b*	Form ²⁾
1	M	34.4 ^{ac}	11.6 ^{ab}	12.9 ^{ab}	R (>M)
	MC	31.8 ^b	12.2 ^a	11.5 ^a	R (>M)
	ML	35.2 ^{ad}	11.3 ^b	12.6 ^{ab}	R (>M)
	MCL	31.8 ^{bc}	13.8 ^{ab}	13.0 ^{abc}	R (>M)
	MCLN	34.4 ^{ac}	7.3 ^c	13.9 ^{bc}	M
	MCN	32.1 ^b	8.2 ^{cd}	12.6 ^{abc}	M
	MLN	36.2 ^d	8.0 ^{cd}	14.8 ^{abc}	M
	MN	36.2 ^d	8.1 ^d	14.3 ^c	M
12	M	35.7 ^{ae}	15.4 ^{ac}	8.7 ^a	R
	MC	32.5 ^{acd}	15.4 ^a	6.6 ^a	R
	ML	37.9 ^b	12.6 ^b	8.7 ^a	R (>>M)
	MCL	32.0 ^{acd}	14.6 ^c	7.8 ^a	R
	MCLN	32.9 ^c	24.6 ^d	11.7 ^b	NO
	MCN	30.9 ^d	25.3 ^e	12.1 ^b	NO
	MLN	37.8 ^b	24.1 ^d	13.1 ^c	NO
	MN	35.7 ^e	26.2 ^e	15.0 ^d	NO
24	M	37.9 ^{ae}	14.5 ^{ac}	10.1 ^a	R
	MC	33.6 ^b	15.1 ^a	7.5 ^b	R
	ML	36.7 ^{ac}	12.5 ^b	10.0 ^a	R (>>M)
	MCL	34.6 ^b	12.9 ^{bc}	10.0 ^a	R
	MCLN	35.3 ^{bc}	24.1 ^{df}	13.9 ^c	NO
	MCN	34.6 ^b	26.5 ^e	15.1 ^d	NO
	MNL	39.6 ^d	23.3 ^d	13.1 ^e	NO
	MN	38.2 ^e	24.4 ^f	14.5 ^d	NO

The different superscript letter within column and line are significantly different ($p < 0.05$) for triplicate samples.

¹⁾ Symbols are same as shown in Table 2.

²⁾ Myoglobin forms, R: reduced form, M: met form, NO: nitric oxide form. Minor form is shown in the parenthesis.

L* value was higher than in non-fermented meat. That was due to the decrease in the meat pH resulting in high lightness¹⁵⁾.

In intact meat, the a* value increased during fermentation, due to the formation of reduced myoglobin. These results corresponds to the increase in reduced myoglobin under anaerobic condition¹³⁾.

In cured meat the a* value just after addition of nitrite was lower than intact meat and increase sharply during fermentation. The color changes are due to the formation of metmyoglobin and followed by nitric oxide myoglobin¹⁴⁾. Both intact and cured meat prepared with chitosan showed higher a* value. The higher formation of reduced myoglobin by chitosan might be due to direct reducing action of myoglobin or the activation of metmyoglobin reductase in meat.

In conclusion, chitosan could protect fermented meat from bacterial spoilage, repress lipid oxidation, dissipate residual nitrite and result in better of color.

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発酵肉の性状に及ぼすキトサンおよび亜硝酸塩の影響

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牛肉にキトサンを添加して、腐敗細菌の抑制効果を比較した。キトサンは、Micrococci, Staphylococci, Pseudomonades, Coliform の順に、経時的にキトサン濃度が高いほど抑制した。キトサン, *Lactobacillus plantarum* スターター, 亜硝酸塩を組合せて添加し、30℃で発酵中の Gram 陰性菌数, 脂質酸化 (TBA 値), 残留亜硝酸根, 色調の各性状について比較した。キトサン添加および *Lactobacillus plantarum* 接種によって、TBA 値は 30% および 8%, 残留亜硝酸根は 52% および 60% に各々低下し、そして Gram 陰性菌数は 1/10 に抑制された。亜硝酸塩の添加で TBA 値が低くなり、色調はよくなったが、Gram 陰性菌は抑制されなかった。キトサン, *Lactobacillus plantarum* および亜硝酸塩を併用すると Gram 陰性菌数は 1/100 に抑制され、TBA 値は 36% に、残留亜硝酸根は 63% に低下し、色調がよくなることが認められた。キトサンは発酵肉の腐敗菌および脂質酸化を抑制するために有用であり、とくにキトサン, *Lactobacillus plantarum* および亜硝酸塩の併用は効果的であった。

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