

Action of isolated *Micrococcus* sp., *Pediococcus* sp. and *Lactobacillus* sp. in fermented dry sausage¹.

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Abstract

Five different commercial fermented sausage (made in Japan, Germany and Italy) were sampled and used for the isolation of the starter culture.

This study, therefore, would like to evaluate the effect of using *Micrococcus* sp., *Pediococcus* sp. and *Lactobacillus* sp. used, singly, on fermented sausage characteristics.

Bacterial growth of all treatments including the control gradually increased up to the 14th day and remained almost constant until the end the 21th days of ripening.

At the last day of ripening, *Micrococcus* sp. inoculated sausage gave the highest aminonitrogen followed by the control. On the last day of ripening, redness values of the three inoculated sausage were essentially similar and higher than the control.

All the TVBN values, however, are still within the acceptable TVBN range.

Sausages inoculated with *Micrococcus* sp. obtained the highest odor and flavor scores.

General acceptability scores were also higher in inoculated sausage.

Key words : fermented sausage, starter culture, ripening, amion-nitrogen.

Introduction

For centuries fermented sausages has been produced, initially as a means of Preservation and more recently for its particular flavor (Eversen et al, 1970). Product preservation and flavor development is brought about by the action of microorganisms, known as starter cultures, on meat components and the proper fermentation

process. Among the many stater cultures being used in the manufacture of fermented sausages, species of *Pediococci*, *Lactobacilli* and *Micrococci* are the most widely used.

Lyophilized cells of *Pediococcus cerevisiae*, which is now known as *Pediococcus acidilactici*, on a dextrose carrier was the very first commercial starter culture offered to the sausage industry (Deibel and Niven, 1957). Since then, many

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studies on the use of several microorganisms and their effects on processed meat have been done. Bartholomew and Blumer (1977) used commercial *Pediococcus cerevisiae* as starter culture for country style hams and found that the total time required to produce aged hams may be shortened by the inoculation of *P. cerevisiae*. Raccach (1989) found out that LACTACEL 75, a selected strain of *Pediococcus* sp., controlled the growth of *S. aureus* in the outer surface (0.1–0.5 cm, depth) of both Genoa sausage and pepperoni during the fermentation period to attain pH 5.0. Daly et al (1973) studied the effect of different starter cultures that consisted of *Lactobacillus-Micrococcus* combination (Niskanen and Nurmi, 1976) and suggested that *Micrococcus* species were responsible for the inhibition of enterotoxin production. Christiansen et al (1975) demonstrated that the pH drop resulting from the fermentation of glucose was the most important factor controlling botulism toxin in fermented sausages. Vignolio et al (1989) observed that Argentina salami produced with three selected groups of two strains each of *Lactobacillus plantarum* and *Micrococcus varians* required from seven to eight days to reach the same organoleptic characteristics as those produced with traditional techniques, requiring 14 to 15 days for the ripening process to be completed.

The presence of microorganisms during the fermentation process can either be due to chance and random inoculation of "wild" microorganisms from the environment (Bacus, 1984) or through inoculation of desirable microorganisms. Most starter inocula that are being used, however, are commercial-type mixtures of different microorganisms. This study, therefore, would like to evaluate the effect of using *Micrococcus* sp., *Pediococcus* sp. and *Lactobacillus* sp. used, singly, on fermented sausage characteristics.

Materials and Methods

Isolation of starter cultures

Five different commercial fermented sausage (made in Japan, Germany and Italy) were sampled and used for the isolation of the starter culture. Appropriate dilutions were then made and 1 ml of sample was plated on Standard Methods Agar (Eiken) for isolation of common bacteria and another 1 ml on Lactobacillus culture agar for the isolation of lactic acid bacteria. All the plates were incubated for 48 hours at 30°C. Representative colonies from each plate were then picked up and streaked and stabbed on agar slants. The slants were also incubated for 48 hours at 30°C. The colonies were then smeared and fixed on glass slides, Gram's stained and examined under the microscope. Mixed cultures were purified by streaking on previously solidified agar plates.

Similar isolates from all the sausages samples were grouped together and their percentages were calculated. Groupings of the same isolates were done on the basis of their morphological characteristics (color on agar slants, size, form of growth, etc.), Gram's staining and catalase reactions by hydroxy peroxide.

Identification of species of *Micrococcus*, *Lactobacillus* and *Pediococcus* were carried out using standard physiological tests according to the Manual of Methods for General Bacteriology (1981) and Bergey's Manual for Determinative Bacteriology, 8th Edition.

Preparation of starter culture

One loopful of culture, each from agar slants of purified *Micrococcus* sp., *Lactobacillus* sp., respectively, were transferred to tubes containing 10 ml of Tryptic Soy Broth (TSB, Eiken). The tubes were incubated at 30°C for 48 hours. Each inoculation TSB was then poured to a flask containing a liter of TSB. The flasks were then incubated at 30°C for 5 days. After 5 days, the cultures were harvested by centrifugation at 5°C using

5,000r. p. m. for 20 minutes. After centrifugation, the supernatant was discarded and the paste-like precipitate was washed with a small amount of saline, transferred to another flask and kept inside the refrigerator until use.

Fermented sausage preparation

Lean meat of pork was trimmed of all visible connective tissues. It was then cut into cubes ground using an electric grinder. The curing ingredients and species consisting of 1.9% sodium chloride, 1.5% sugar, 0.3% glucose, 0.1% NaNO_3 / NaNO_2 , 0.3% white pepper, 0.1% Allspice and 0.1% Mace were applied to the ground meat. Except for NaNO_3 / NaNO_2 which was dissolved in a small amount of water prior to application, all ingredients were applied in their dried form. The meat and the ingredients were mixed thoroughly with a wooden spatula which was previously immersed in hot water. After mixing, the meat mixture was divided into 4 equal portions. One at a time, *Micrococcus*, *pediococcus* and *Lactobacillus* starter cultures were mixed with the first, second and third portions, respectively. The fourth portion did not receive any inoculum and served as the control. Immediately after inoculation, the mixture was stuffed in Naturin casings (40 mm, diameter), linked to about 15 cm long and labeled. The uninoculated sample was likewise stuffed and linked. All treatments were then hung inside an incubator set at 20°C, 90 % RH for 2 days. After 2 days, the temperature and relative humidity of the incubator were lowered to 10°C and 30%, respectively. All sausage links were ripened at these conditions up to 21 days.

Bacterial count

Ten gram sample was transferred to 90 ml sterile saline and homogenized. One milliliter of sample was plated onto Standard Methods Agar plates. All the plates were incubated at 30°C for 48 hours.

pH measurement

The pH of the fermented sausage was determined using the TDA pH Meter Model HM40S.

pH measurement was done at 3, 7, 14, and 21 days.

Amion nitrogen analysis.

Amion nitrogen contents of fermented sausages were determined by Formol titration method.

Total volatile basic nitrogen (TVBN) analysis

Ten gram meat sample from each treatment was weighed and homogenized with 90 ml distilled water. Into the homogenate, 10 ml of 20% TCA was added and it was allowed to stand of 15 minutes. It was then filtered using ADVANTEC filter paper No. 5 C. To determine TVBN, micro-diffusion plates of CONWAY's unites were used.

Color

Hunter L, a and b values were determined for all treatmets at time intervals of 3, 14 and 21 days using JASCO UBest-50UV/VIS Spectrophotometer.

Moisture content

After 21 days ripening periodm, moisture content of all the samples were determined following the procedures set by AOAC (1975).

Water holding capacity

Water holding capacity of the samples were measured after 21 days of ripening period. It was determined for all treatments using the Pressed Method (30 kg press per 1 g of sample). Six pieces of filter paper (ADVANTEC No. 2, 70mm) was weighed and the weight was recorded. Using a nylon filter paper, about 1.0 gram meat sample was weighed. The nylon filter paper with the sample was then placed between the six pieces of filter papers and pressed using the Marubishi Press at 30 kg pressure for 1 minute. After pressing, the nylon filter plus the sample were discarded and the six pieces of filter papers were again weinghed. Water holding capacity was then calculated as % Free Water using the formula below :

$$\text{Free Water} = \frac{\left(\begin{array}{c} \text{weight of filter} \\ \text{after pressing} \end{array} \right) - \left(\begin{array}{c} \text{weight of filter} \\ \text{before pressing} \end{array} \right)}{\text{sample weight}} \times 100$$

(%)

Texture and firmness

Textural properties of the sausages were determined by cutting sausage samples of each treatment into approximately $1 \times 1 \times 1$ cm. Each sample was then cut using the Rheometer machine (Fudōkogyō k. k.). Softness or firmness of the sausages were determined by getting the force needed to cut the $1 \times 1 \times 1$ cm meat sample at the center. Higher force values indicate higher firmness of the meat.

Sensory evaluation

Exactly one week after the last day of ripening period, the sausages were subjected to sensory evaluation. The sausage from every treatment were sliced into approximately 2 mm thick and randomly served for evaluation to the students and staff of the Institute of Meat Preservation using a fivepoint quality scale scoring. The sausages were evaluated for their flavor, tenderness, sourness, odor and general acceptability.

Results and discussion

Microflora of commercial, fermented sausage samples.

Table 1 shows the percent microflora of the fermented sausages where the starter cultures used in this experiment were obtained. In the Standard Methods Agar, *Pediococcus* species predominated, followed by the coagulase-negative Micrococcaceae, Lactobacilli and Micrococci. In

the *Lactobacillus* culture agar, almost the same types of microorganisms appeared except for Micrococci. *Lactobacillus* species were predominantly isolated followed by *Pediococcus* species. Fermented, dry sausages inoculated with isolated cultures of *Lactobacillus*, *Pediococcus* and *Micrococcus*.

Table 1. Percent composition of microflora of commercial fermented sausages. (%)

Microorganism	Isolation microorganisms	
	Standard Methods Agar Plates	Lactobacillus Culture Agar Plates
<i>Micrococcus</i> sp.	9.29	—
<i>Pediococcus</i> sp	41.88	34.21
<i>Lactobacillus</i> sp	18.60	63.16
Coagulase-negative Micrococcaceae	30.23	2.63

Bacterial count. Bacterial count of fermented, dry sausages inoculated with species of *Micrococcus*, *Pediococcus* and *Lactobacillus* is presented in Figure 1. At day 0, initial level of bacteria in the inoculated samples ranged from 4.0×10^6 to 4.0×10^7 CFU/g, with *Pediococcus*-inoculated sample obtaining the highest count. Bacterial counts on *Micrococcus* and *Lactobacillus* treatments were almost the same day 0 while the control gave a relatively low bacterial count (8.0×10^3).

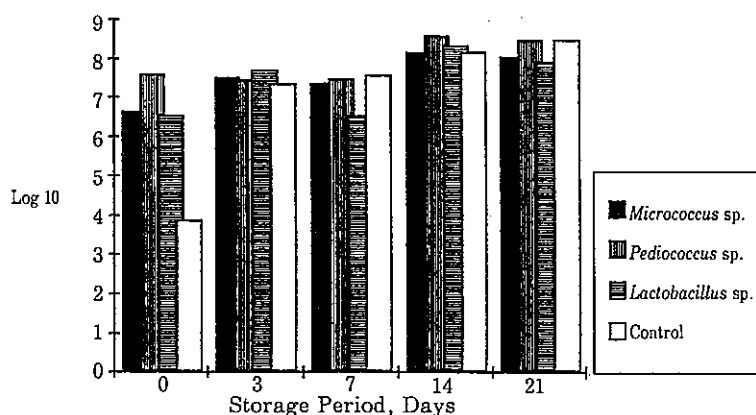


Figure 1. Bacterial count of fermented, dry sausages inoculated with genus name of *Micrococcus*, *Pediococcus* and *Lactobacillus*.

Bacterial growth of all treatments including the control gradually increased up to the 14th day and remained almost constant until the end of the 21 days of ripening. It can also be observed that among the three starter cultures used, *Pedio-coccus* species almost always gave the greater growth count.

pH. The pH values of the fermented, dry sausages inoculated with starter cultures is presented in Table 2. *Micrococcus*-inoculated sausages the highest pH values at all predetermined ripening intervals. These values almost similar to the values measured in the control. These results are quite expected since micrococci, compared with the other two starter cultures used, do not produce as much acid so as to lower pH of the mixture. Bacus (1984) stated that *Micrococcus* sp. are poor producer of lactic acid thus usually used in combination with a "lactic acid producing, meat fermenting bacteria". pH values of sausages inoculated with *Pediococcus* sp. and *Lactobacillus* sp. on the third day of ripening are similar. However, from the 7th up to the last day of ripening, *Pediococcus*-inoculated sausages gradually gave increased pH values while those inoculated with *Lactobacillus* sp. decreased the pH of the sausages. These results seem to show the ability of *Lactobacillus* sp. used in the experiment to produce more acid hence lowering meat pH in a shorter time compared with those of the control and the other two starter culture - inoculated sausages.

Table 2. pH values of fermented, dry sausages inoculated with Genus name of *Micrococcus*, *Pediococcus* and *Lactobacillus*.

Treatment	pH			
	Days			
	3	7	14	21
<i>Micrococcus</i> sp.	5.30	5.47	5.49	5.57
<i>Pediococcus</i> sp.	5.02	5.08	5.11	5.17
<i>Lactobacillus</i> sp.	5.03	4.85	4.81	4.90
Control	5.18	5.14	5.19	5.25

Amion-nitrogen content. The amion-nitrogen contents (mg) of the control and inoculated dry, fermented sausages are shown in Table 3. At the third day of ripening, lowest amion-nitrogen was observed in the control. *Lactobacillus* sp. -treated sausages had slightly lower amion - nitrogen content compared with the other two sausages inoculated with *Micrococcus* sp. and *Pediococcus* sp. All amounts, however, increased as ripening period lengthened for all treatments. At the last day of ripening *Micrococcus*-inoculated sausages gave the highest amino-nitrogen followed by the control. Amounts of amion-nitrogen are similar in both sausages with *Pediococcus* sp. and *Lactobacillus* sp. These results may be explained by the amount of proteolysis that occurred during ripening. The higher amount of amion-nitrogen in *Micrococcus*-inoculated sausages may be due to the ability of these organisms to exhibit proteolysis on proteins since *Micrococcus* specifically *M. Varians* has gelatinase activity (Kocur, 1986). DeMasi et al (1990) reported that the total free amino acid concentration in sausages fermented with *M. varians* present was approximately 11% higher than when *M. varians* was absent. On the other hand, *Pediococcus* sp. which are not regarded as possessing proteolytic activity (Deibel et al, 1961) and *Lactobacillus* sp. both produce acid during ripening. This fact might explain a slightly lower amino-nitrogen for both sausages compared with those of the control and *Micrococcus*-inoculated sausages. Kletter and Baum-

Table 3. Amion nitrogen contents of fermented, dry sausages inoculated with genus name of *Micrococcus*, *Pediococcus* and *Lactobacillus*.

Treatment	amion-nitrogen (mg %)		
	Days		
	3	14	21
<i>Micrococcus</i> sp.	101.76	188.86	304.43
<i>Pediococcus</i> sp.	102.20	190.59	270.80
<i>Lactobacillus</i> sp.	97.45	232.80	267.30
Control	84.95	167.30	301.84

gartner (1980) stated that rapid development minimizes proteolytic activity of bacterial origin and limits the development of overall flavor except acidity. The high amino-nitrogen in the control samples at the last day of storage was unexpected since the control did not contain any inoculum. It is very probable that at the conditions where the sausages were ripened, fermenting and proteolytic bacterial growth were favored. This observation can also be seen in Figure 1 showing the almost same bacterial count in the control as of those with the inoculated sampled during the last day of ripening.

Color. Hunter L, a and b color values of the fermented, dry sausages with or without starter cultures are presented in Table 4. Generally, L (lightness) and b (yellowness) values have a decreasing trend while a (redness) values increased with increasing ripening period for all treatments. Redness color values were lowest in the control sausages until the last day of ripening. This in-

dicates positive action of the starter cultures on cure color development. On the last day of ripening, redness values of the three inoculated sausages were essentially similar and higher than the control.

On the third day of ripening, redness value was highest for *Micrococcus* inoculated sausages. Thereafter, it stabilized until the last day of the ripening period. It is very interesting to note that the redness values of the other three treatments needed at least about 21 days before it increased to approximately the same redness color intensity as those inoculated with *Micrococcus* sp. These results appear to show that in terms of color development in fermented sausages, *Micrococcus* starter culture can achieve a faster color development in a much shorter time than either *Pediococcus* and *Lactobacillus* starter cultures. This may be attributed to the ability of *Micrococcus* species to reduce nitrates to nitrite. Many sausage processors utilize *Micrococcus* sp. specifically for this purpose. Early researchers depended upon chance inoculation by more fermentative bacteria and on the utilization of *Micrococcus aurantiacus* as a starter culture to reduce nitrate to nitrite (Niinivaara, 1955). Even now that nitrite cures are available, many European processors of dry sausages feel that "cure color development" is more effective and more stable when Micrococci are present (Bacus, 1984).

TVBA, WHC, Moisture and Rheometer reading values. Table 5 shows the total volatile basic nitrogen (TVBN) at the last day of ripening and the water holding capacity (WHC) expressed as percent free water, moisture content and Rheometer reading values at 28 days of the fermented, dry sausages.

The highest TVBN (mg%) was measured in the *Lactobacillus*-inoculated sausages. TVBN of *Micrococcus* and *Pediococcus* treated sausages were essentially the same and both values were lower than those of sausages inoculated with *Lactobacillus* sp. The lowest value was observed

Table 4. Hunter L, a and b color values of fermented, dry sausages inoculated with species of *Micrococcus*, *Pediococcus* and *Lactobacillus*.

Treatment	Day after fermentation		
	3	14	21
L* (lightness)			
<i>Micrococcus</i> sp.	54.45	55.85	54.91
<i>Pediococcus</i> sp.	58.06	57.73	56.21
<i>Lactobacillus</i> sp.	58.41	56.75	54.04
Control	57.02	57.18	55.00
a* (redness)			
<i>Micrococcus</i> sp.	7.51	7.11	7.11
<i>Pediococcus</i> sp.	6.47	6.96	7.41
<i>Lactobacillus</i> sp.	6.24	7.00	7.19
Control	6.15	6.78	6.55
b* (yellowness)			
<i>Micrococcus</i> sp.	5.25	4.11	3.47
<i>Pediococcus</i> sp.	4.50	4.25	3.80
<i>Lactobacillus</i> sp.	4.23	3.23	3.13
Control	4.17	3.92	2.95

Table 5. Total volatile basic nitrogen, water holding capacity, percent moisture content and rheometer values of fermented, dry sausages inoculated with genus name of *Micrococcus*, *Pediococcus* and *Lactobacillus*.

Treatment	TVBN (mg%)	Free water(%)	Moisture (%)	Rheometer reading (gram - force / cm ²)
<i>Micrococcus</i> sp.	21.39	2.34	40.40	34.21
<i>Pediococcus</i> sp.	20.37	2.12	38.32	35.60
<i>Lactobacillus</i> sp.	27.16	2.12	38.32	35.60
Control	18.31	2.34	42.62	24.89

in the control sausages. All the TVBN values, however, are still within the acceptable TVBN range. This indicates that at 21 days of ripening, bacteria was not extensive enough as to produce volatile basic nitrogen, a product which is an indication of putrefaction.

The WHC of the sausages after 28 days are very high. The highest value was measured in sausages inoculated with *Lactobacillus* sp. *Micrococcus*-inoculated and control sausages have similar WHC which are slightly lower than those of sausages inoculated with *Pediococcus* sp. Moisture content, on the other hand, were almost similar in the control and *Micrococcus* sp. -inoculated sausages. The highest moisture content was measured in the *Pediococcus* sp. treatment while the lowest was in the sausages with *Lactobacillus* sp. These results seem to show that water molecules were released more uniformly and slowly. Moreover, higher WHC and moisture content of sausages with *Micrococcus* sp. and *Pediococcus* sp. may be related to their ultimate pH (Table 2). It must be noted that the final pH that these sausages obtained in last day of ripening are still way above the isoelectric point of the fibrillar proteins hence more spaces in the dimensional network of the filaments are still available for water molecules to occupy. In contrast, *Lactobacillus*-inoculated sausages obtained a more lower WHC and moisture content. This again may be related to the final pH of the product. Lowering of pH, specifically those pH below the isoelectric point, will result in less water retaining

capacity (Bacus, 1984). Thus, at pH values lower than the isoelectric point, water molecules are more readily released.

The gram-force needed to cut the sausage samples, on the other hand, is higher in inoculated sausages than the control. *Lactobacillus*-inoculated sausages appeared firmer than the sausages with *Micrococcus* sp. Sausages with *Pediococcus* sp. needed lesser gram-force than the sausages with the other starter cultures. The control obtained the lowest force values. These firmness results may be related to the amount of moisture that was left on the sausages and the extent of acid production by the organisms, both of which contributes to the ultimate firmness of the fermented sausages.

Sensory evaluation. Mean sensory scores of fermented, dry sausages with or without starter cultures is shown in Table 6. Sausages inoculated with *Micrococcus* sp. obtained the highest odor scores. Control and *Lactobacillus*-inoculated sausages were given the same scores while sausages with *Pediococcus* sp. obtained slightly lower odor scores in comparison with the other treatments. All odor scores, however, are still in the acceptable normal odor range of fermented sausages.

Tenderness scores ranged from 2.63 for sausages with *Lactobacillus* sp. to 3.25 *Pediococcus*-inoculated sausages. Control sausages were given a score of 3.0 while those of the *Micrococcus*-inoculated obtained a 2.75 average score. The slightly lower tenderness score of *Micrococcus*

Table 6. Mean sensory scores of fermented, dry sausages inoculated with Genus name of *Micrococcus*, *Pediococcus* and *Lactobacillus*.

Quality Characteristic ¹	Inoculated starter culture			Control
	<i>Micrococcus</i> sp.	<i>Lactobacillus</i> sp.	<i>Pediococcus</i> sp.	
Odor	3.75	2.88	2.75	2.88
Tenderness	2.75	2.63	3.25	3.00
Flavor	3.38	3.25	3.12	3.00
Sourness	3.25	3.62	3.50	3.12
Acceptability	3.56	3.38	3.75	3.00

¹Odor ; Tenderness ; Flavor ; General Acceptability :

1-unacceptable; 3-normal; 5-very acceptable

Sourness :

1-weak ; 3-normal ; 5-very strong

and *Lactobacillus* treatments may be due to the amount of moisture content that was left in these sausages at the end of the ripening period. It can be noticed that was the moisture content decreased (Table 5), tenderness scores also decreased.

Micrococcus-inoculated sausages were given the highest scores for flavor. This is in agreement with the results obtained for the amount of amion-nitrogen (Table 3) which may have contributed to the acceptable flavor development in the sausages. Sausages with *Lactobacillus* sp., on the other hand, obtained higher flavor scores than both the control and *Pediococcus*-inoculated sausages. All the flavor scores are within the acceptable flavor range.

Sourness scores of sausages with starter cultures were higher than the control, *Lactobacillus* treatment obtained the highest sourness score followed closely by *Pediococcus*. These results are quite expected since these two cultures are producing acid hence contributing to the sour taste of the sausages. *Micrococcus*-inoculated sausages were slightly sour while control sausages obtained the normal sour taste of the fermented sausages.

General acceptability scores were also higher in inoculated sausages. Overall acceptability scores were higher in *Pediococcus*-inoculated

sausages which is slightly higher than the acceptability scores of sausages with *Micrococcus* sp. and *Lactobacillus* sp. The control samples obtained the lowest acceptability scores, nevertheless, it still falls in the normal acceptable range.

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diococcus sp. および *Lactobacillus* sp. を再び醗酵ソーセージに単用で摂取した時のソーセージの品質に対する影響についてしらべた。

微生物は各区とも14日まで増殖し、21日目の熟成終了時まで生残した。

熟成末期のアミノ態Nの蓄積は *Micrococcus* 接種区が最も高く、次いで対照区であった。

熟成末期の赤色度は概して3接種区とも対照区よりも高かった。各区ともTVBN値は高いが許容できる範囲であった。 *Micrococcus* sp. 接種区は匂い及び風味において官能的に高い評価が得られた。総合的な官能評価は概して微生物スターター接種ソーセージが高かった。

醗酵ソーセージにおける分離 *Micrococcus* sp., *Pediococcus* sp. および *Lactobacillus* sp. の活性。

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摘 要

5種類の市販醗酵ソーセージ(日本国, ドイツおよびイタリー産)から分離した *Micrococcus* sp., *Pe*

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