

Bioactive amines changes in raw and sterilised milk inoculated with *Pseudomonas fluorescens* stored at different temperatures

M BEATRIZ A GLORIA,^{1*} PATRICIA RL SARAIVA,¹ JULIANA C S RIGUEIRA² and SEBASTIÃO C C BRANDÃO²

¹LBqA – Laboratório de Bioquímica de Alimentos, FAFAR, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Belo Horizonte, MG, 31270-901, Brazil and ²Laboratório de Análise de Alimentos, DTA, Universidade Federal de Viçosa, Viçosa, MG, 36540-000, Brazil

The presence of bioactive amines in raw and sterilised milk inoculated with *Pseudomonas fluorescens* during storage at 4°C, 7°C and 10°C for 6 days was investigated. Spermine, spermidine, putrescine, serotonin and phenylethylamine were present in the samples immediately after milking. Histamine, cadaverine and tyramine were formed in the raw milk on the 4th day of storage at 10°C, increasing significantly afterwards. Cadaverine was formed during sterilisation. There was no significant change in amines, acidity, thermostability and alizarol tests throughout storage of the sterilised milk; however, a putrid smell was detected at every temperature on the 6th day. Therefore, raw or sterilised milk storage at 4–7°C should not exceed 4 days. Furthermore, raw milk should not be stored at 10°C.

Keywords Bioactive amines, Refrigerated storage, *Pseudomonas fluorescens*, Proteolysis.

INTRODUCTION

The use of bulk chillin tanks to store milk at dairy farms and plants under refrigerated conditions was imposed by Brazilian law in 2002 at temperatures up to 7°C and 10°C, respectively (Brasil 2002). Therefore, today's milk production and marketing practices result in a raw product that can be stored under refrigeration for up to 4–5 days before processing. The system proved to be efficient, economic and labour saving. Furthermore, the growth of pathogenic mesophilic micro-organisms decreased significantly. However, with the introduction of chilled storage of raw milk, the growth of psychrotrophic micro-organisms has increased and the problem of milk spoilage increased, affecting the shelf-life, flavour, texture, quality and yield of several dairy products (Wang and Jayarao 2001; Arcuri *et al.* 2008; Mu *et al.* 2009).

The chilled storage of milk before processing can allow the growth of psychrotrophic micro-organisms (Dufour *et al.* 2008), which are likely to dominate the microbiota at refrigerated temperatures. Most psychrotrophs would not be a serious problem in milk because they are eliminated by conventional milk pasteurisation or UHT

treatments. However, they can produce proteases and lipases which survive sterilisation processes and affect the quality of milk and dairy products. Among psychrotrophic micro-organisms detected in milk, *Pseudomonas fluorescens* is the most numerous and fastest growing bacteria and display strong proteolytic activity (Kives *et al.* 2005; Arcuri *et al.* 2008; Mu *et al.* 2009; Nörnberg *et al.* 2009).

The proteolytic enzymes from psychrotrophic micro-organisms can cause destabilisation of casein micelles, gelation of UHT milk, development of bitter and putrid flavours, liberation of amino acids, and decreased dairy products yields (Kives *et al.* 2005; Dufour *et al.* 2008; Mu *et al.* 2009; Nörnberg *et al.* 2009). The higher protein hydrolysis will correspond to an increased release of amino acids which will lead to the formation of biogenic amines (Santos *et al.* 2003; Gloria 2005; Kalac and Krausová 2005). However, information is scarce regarding the influence of psychrotrophic micro-organisms and its proteolytic activity on the formation of bioactive amines in milk and dairy products. Therefore, the objective of this study was to investigate if *P. fluorescens* could affect the production of biogenic amines of raw and sterilised

*Author for correspondence. E-mail: mbeatriz@ufmg.br

milk during cold storage at different temperatures. Furthermore, the profile and levels of amines in bulk milk immediately after milking and in sterilised milk were investigated for the first time.

MATERIALS AND METHODS

Samples and reagents

Raw milk from bulk tanks were obtained at the dairy farm of Federal University of Viçosa, Viçosa, MG, Brazil, in the summer of 2003, immediately after milking in three nonconsecutive days ($n = 3$). Young healthy pure Dutch Belted dairy cows were submitted to mechanical milking practices under sanitary and hygienic standards. Milk samples were collected in refrigerated bulk tanks prior to transportation to the dairy plant for processing. The samples were analysed for bioactive amines, proteolytic activity, sensory evaluation and also to microbial and physicochemical characteristics according to Brazilian legislation.

For each of the three experiments, the samples were divided into eight lots (8×1 L). Half was sterilised at $121^\circ\text{C}/15$ min using a vertical autoclave (model AV; Phoenix, Araraquara, SP, Brazil). Both raw and sterilised milk samples were inoculated with *P. fluorescens* (ATCC 13525). The pure culture was activated in nutrient broth at $26^\circ\text{C}/48$ h, and replicated in sterilised ($121^\circ\text{C}/15$ min) skimmed milk for 48 h. After dilutions, the culture was inoculated into the samples in order to provide counts of 10^3 cfu/mL. The inoculated samples were stored at 4°C , 7°C and 10°C in biochemical oxygen demand (BOD) incubators (model 347 CD; FANEM[®], Guarulhos, SP, Brazil) for up to 6 days. Within 48 h intervals, samples were collected and analysed for the levels of bioactive amines, psychrotrophic bacteria counts, titratable acidity, alizarol, thermostability, proteolytic degree and sensory analysis. Each analysis was performed in duplicate.

All reagents were of analytical grade, except HPLC reagents which were LC grade. Ultrapure water was obtained from Milli-Q (Millipore Corp., Milford, MA, USA). Bioactive amines standards were purchased from Sigma Chemical Co. (St Louis, MO, USA). They included putrescine (PUT) dihydrochloride, spermidine (SPD) trihydrochloride, spermine (SPM) tetrahydrochloride, agmatine (AGM) sulphate, cadaverine (CAD) dihydrochloride, serotonin (SRT) hydrochloride, histamine (HIM) dihydrochloride, tyramine (TYM), tryptamine (TRM) and 2-phenylethylamine (PHM) dihydrochloride. Plate Count Agar

was purchased from Biobras (Montes Claros, MG, Brazil).

Analytical methods

Bioactive amines

The amines were determined by ion-pair HPLC and detected fluorimetrically after post-column derivatisation with *o*-phthalaldehyde (Santos *et al.* 2003). The amines were extracted from the samples with 1.2 g of sulphosalicylic acid. A Shimadzu LC-10AD system connected to a RF-551 fluorimetric detector at 340 nm and 445 nm of excitation and emission, respectively, were used (Kyoto, Japan). The columns used were a reversed-phase μ Bondapak C18 column (300×3.9 mm i.d., $10 \mu\text{m}$) and a μ Bondapak C18 guard-pak insert (Waters, Milford, MA, USA). The mobile phases were: (A) solution of 0.2 mol/L sodium acetate and 15 mmol/L 1-octanesulphonic acid sodium salt, adjusted to pH 4.9 with acetic acid, and (B) acetonitrile. The flow rate was 0.8 mL/min and the gradient was time (min)/%B: 13/11; 19/26; 24/11; 45/11. The post-column derivatisation reagent was delivered at 0.4 mL/min. The levels of amines were calculated by direct interpolation in a calibration curve.

Mesophilic and psychrotrophic micro-organisms

Total plate counts of mesophilic and psychrotrophic micro-organisms were determined according to APHA (1992) before and after inoculation of *P. fluorescens* to the milk samples, respectively.

Physicochemical characteristics

The physicochemical characteristics were determined using the methods established by the Brazilian legislation. Density, titratable acidity, fat content, alizarol test and pH were performed according to Lanara (1981). The cryoscopic index was determined according to AOAC (1995) using a digital electronic cryoscope (Laktron 512-L; Laktron Indústria Eletrônica Ltda, Londrina, PR, Brazil).

Proteolytic degree

The proteolytic degree was calculated by determining the ratio of whey protein to total milk protein. The fractions were separated and quantified by gel filtration HPLC (Lanara 1981). Ten millilitres of 24% trichloroacetic acid was added to 20 mL sample under agitation. The samples were kept at $25.0 \pm 0.5^\circ\text{C}$ for 1 h, filtered through filter paper and through $0.45 \mu\text{m}$ pore diameter membrane (Millipore, Milford, MA, USA).

The chromatographic system included a CG-480C (CG Instrumentos Científicos, São Paulo, SP, Brazil) liquid chromatograph equipped with a Reodyne model 7125 sample injector, and Zorbax GF-250 (Dupont) gel filtration column (250 × 9.4 mm i.d., 5 µm) preceded by pre-column (40 × 2 mm i.d.) of the same packing material. A CG Instrument model 350 variable wavelength detector was used to monitor the column eluate at 205 nm. An isocratic mobile phase (1.74 g K₂HPO₄ and 12.37 g KH₂PO₄ in 1 L HPLC water, pH 6.0) was used at a flow rate of 1.0 mL/min. The concentrations of whey protein and casein were calculated by direct interpolation in an analytical curve prepared with milk containing known amounts of whey.

Thermostability

For the determination of the resistance of milk to heat treatment, the samples were placed in a water bath at 85°C. The absence of coagulation indicated stability to heat, providing a negative result (Pinheiro and Mosquim 1991).

Sensory evaluation

The samples were evaluated by a five member trained descriptive sensory panel. The panellists were further trained with respect to definitions and intensities of milk smell attributes (Claassen and Lawless 1992).

Statistical analyses

The results were submitted to analysis of variance (ANOVA) and the means were compared by the Duncan and Mann–Whitney tests at 5% probability. The existence of correlation between the

investigated parameters was determined by Pearson correlation at 1% probability (Pimentel-Gomes 2009).

RESULTS AND DISCUSSION

Characteristics of the milk immediately after milking

The physicochemical, microbial and sensory characteristics of the raw milk from bulk tanks immediately after milking are indicated in Table 1. The density, cryoscopic index, titratable acidity, fat content, alizarol test and mesophilic counts are in accordance with the Brazilian legislation. The mean pH of the samples was similar to values reported in the literature – 6.5 to 6.7 (Pinheiro and Mosquim 1991). Similar initial counts for mesophilic micro-organisms were reported by Arcuri *et al.* (2008). Proteolysis was not detected. Descriptive sensory evaluation indicated that the smell of the milk was characteristic of fresh milk. Therefore, all milk samples met the standards outlined in the technical regulation of production, identity and quality of type B milk (Brasil 2002) which is determined by the fat content and by the microbial counts.

The types and levels of amines present in milk immediately after milking are indicated in Table 2. Among the ten amines investigated, five were detected – SPM, SPD, PUT, SRT and PHM. Spermine, SPD and PHM were present in every sample analysed. Spermine was the most prevalent amine, corresponding to 36% of total amine levels. Sang-uansermisri *et al.* (1974) and Santos *et al.* (2003) also detected SPM and SPD in cows' milk. The presence of these amines in milk is important as

Table 1 Physicochemical, microbial and sensory characteristics of the raw milk from bulk tanks immediately after milking

Characteristics (units)	Mean ± standard deviation ^a	Legislation (Brazil, 2002)
Density (g/mL)	1.032 ± 0.004	1.028–1.034
Cryoscopic index (°H)	–0.530 ± 0.005	Maximum – –0.530
Titratable acidity (g lactic acid/100 mL)	0.18 ± 0.01	0.14–0.18
Fat content (g/100 g)	3.6 ± 0.2	Minimum 3.0
Alizarol test	Red colour, no coagulation	Red colour, no coagulation
Mesophilic count (cfu/mL)	8.1 × 10 ⁴	Maximum 5 × 10 ⁵
pH	6.54 ± 0.00	^b —
Proteolytic degree (%)	0	—
Smell ^c	Characteristic	—

^an = 6.

^b— Not mentioned in the legislation.

^cConsensus of a five member descriptive trained panel.

Table 2 Levels of bioactive amines in milk immediately after milking

Amines	Levels (mg/L) ^a			CV (%) ^b
	Minimum	Maximum	Mean	
Spermine	0.13	0.26	0.20	32.3
Spermidine	0.01	0.16	0.09	89.3
Putrescine	Nd ^c	0.21	0.10	103.0
Serotonin	Nd	0.28	0.09	173.2
Phenylethylamine	0.04	0.17	0.08	90.7
Total			0.57	99.4

^a*n* = 6; mean levels were calculated by using Nd equal to 0.

^bCV, coefficient of variation.

^cNd = not detected; <0.010 mg/L.

they have functional properties: they play important role in growth, synthesis of DNA, RNA and proteins, and membrane stability (Kalac and Krausová 2005). The types and levels of the other amines found are different from reported data. However, no detail was provided regarding the history or characteristics of the samples used by Sanguansermisri *et al.* (1974); whereas Santos *et al.* (2003) worked with reconstituted dried milk. Therefore, the amines were described for the first time in milk immediately after milking.

Influence of storage temperature on bioactive amines and on the quality of raw and sterilised milk inoculated with *Pseudomonas fluorescens*

Immediately after sterilisation of the raw milk, the presence of CAD was detected in the milk samples. Its formation could be the result of the thermal decarboxylation of lysine during the sterilisation process (Gloria 2005).

During storage of the sterilised milk inoculated with *P. fluorescens*, there was no significant change in the profile and levels of the amines with storage temperature and time, indicating that under the investigated conditions, *P. fluorescens* did not produce amines.

Similar results were observed for the raw milk inoculated with *P. fluorescens* stored at 4°C and 7°C. However, during storage at 10°C, CAD, HIM and TYM were detected on the 4th storage day, resulting in a significant increase in total amine levels. Furthermore, there was a significant increase on the levels of these amines on the 6th day of storage (Figure 1) reaching levels of 8.42, 6.50 and 2.85 mg/L, respectively.

Among the amines formed, CAD and HIM could impart a putrid and pungent flavour to milk; however, no threshold level is described for these

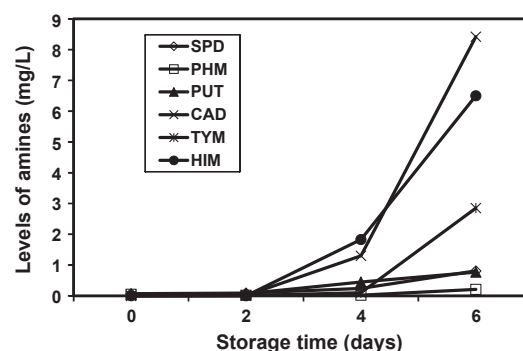


Figure 1 Levels of bioactive amines (mg/L) in raw milk inoculated with *Pseudomonas fluorescens* during storage at 10°C (SPD, spermidine; PHM, phenylethylamine; PUT, putrescine; CAD, cadaverine; TYM, tyramine; HIM, histamine).

amines in milk. Histamine and TYM are involved in adverse effects to human health. Histamine can cause HIM poisoning with symptoms such as erythema, rash and headache, whereas TYM can cause migraine headache. Tyramine can also trigger hypertensive crisis in individuals under monoamine oxidase inhibitor drugs (Gloria 2005). The levels of these amines were not high enough to cause adverse effects; however, they could accumulate to toxic levels on the dairy products produced. Furthermore, these amines are resistant to heat and will not be degraded during thermal processing.

Amines were not produced in the sterilised milk inoculated with *P. fluorescens* and stored at 10°C; therefore, the amines detected in the raw milk probably resulted from natural lactic acid bacteria present, which could have grown at 10°C. The production of amines was affected by storage temperature, with higher amounts found in samples stored at 10°C compared to 4°C and 7°C. To

prevent amine formation, the temperature in bulk tanks at farms should not exceed 4°C.

The counts of psychrotrophic micro-organisms in the raw and sterilised milk after inoculation with *P. fluorescens* were 10^4 and 10^3 cfu/mL, respectively (Table 3). Based on these results, the raw milk contained also some natural psychrotrophic micro-organisms. According to the literature (Kives *et al.* 2005; Nörnberg *et al.* 2009), *P. fluorescens* is the fastest growing bacteria in cold raw milk, however, other psychrotrophs are commonly found. Raw milk is usually also rich in lactococci. Both *Pseudomonas* and *lactococci* have been reported to dominate the microbiota of bulk tank milk.

During storage, there was a significant increase in psychrotrophic counts at every storage

temperature, with faster rates at higher temperatures. The first order reaction rates were 0.772 ($r^2 = 0.9383$), 1.413 ($r^2 = 0.9535$) and 2.605 ($r^2 = 0.7980$) for raw milk and 0.752 ($r^2 = 0.8674$), 1.351 ($r^2 = 0.9890$) and 2.436 ($r^2 = 0.8028$), for the sterilised milk, during storage at 4°C, 7°C and 10°C, respectively. The rate of increase in psychrotrophic bacteria counts was faster in raw compared to sterilised milk.

During storage at 4°C and 7°C, there was no significant increase in acidity with storage time for both raw and sterilised milk inoculated with *P. fluorescens* (Table 4). Similar results were observed for the sterilised milk stored at 10°C. However, a significant increase in acidity was observed for the raw milk during storage at 10°C.

Table 3 Counts of psychrotrophic micro-organisms in raw and sterilised milk inoculated with *Pseudomonas fluorescens* during storage at different temperatures

Milk/storage time (days)	Microbial counts (log cfu/mL) during storage at		
	4°C	7°C	10°C
Raw milk			
0	4.04 ± 0.06 ^{c,x}	4.23 ± 0.11 ^{d,x}	4.00 ± 0.18 ^{d,x}
2	4.78 ± 0.74 ^{bc,z}	6.16 ± 0.00 ^{c,y}	10.5 ± 0.00 ^{c,x}
4	5.13 ± 0.92 ^{ab,z}	7.81 ± 0.00 ^{b,y}	11.5 ± 0.00 ^{b,x}
6	6.50 ± 0.69 ^{a,z}	8.39 ± 0.00 ^{a,y}	12.5 ± 0.00 ^{a,x}
Sterilised milk			
0	3.73 ± 0.07 ^{d,x}	3.91 ± 0.07 ^{d,x}	3.82 ± 0.07 ^{d,x}
2	4.23 ± 0.07 ^{c,z}	5.45 ± 0.07 ^{c,y}	9.82 ± 0.07 ^{c,x}
4	4.52 ± 0.07 ^{b,z}	6.69 ± 0.07 ^{b,y}	10.4 ± 0.07 ^{b,x}
6	6.14 ± 0.07 ^{a,z}	7.91 ± 0.07 ^{a,y}	11.8 ± 0.07 ^{a,x}

Mean (±standard deviation) with the different superscript in the same column (abcd) and in the same line (xyz) for each type of milk are statistically different (Mann–Whitney test, $P < 0.05$).

Table 4 Titratable acidity (expressed as lactic acid) in raw and sterilised milk inoculated with *Pseudomonas fluorescens* during storage at different temperatures

Milk/storage time (days)	Titratable acidity (g lactic acid/100 mL) during storage at		
	4°C	7°C	10°C
Raw milk			
0	0.170 ± 0.001 ^{a,x}	0.170 ± 0.001 ^{a,x}	0.170 ± 0.001 ^{c,x}
2	0.173 ± 0.002 ^{a,x}	0.177 ± 0.002 ^{a,x}	0.190 ± 0.003 ^{bc,x}
4	0.183 ± 0.002 ^{a,y}	0.187 ± 0.002 ^{a,y}	0.230 ± 0.007 ^{b,x}
6	0.187 ± 0.002 ^{a,y}	0.187 ± 0.002 ^{a,y}	0.310 ± 0.002 ^{a,x}
Sterilised milk			
0	0.173 ± 0.001 ^{a,x}	0.173 ± 0.001 ^{a,x}	0.173 ± 0.001 ^{a,x}
2	0.187 ± 0.002 ^{a,y}	0.187 ± 0.002 ^{a,y}	0.193 ± 0.003 ^{a,x}
4	0.187 ± 0.002 ^{a,x}	0.193 ± 0.003 ^{a,x}	0.193 ± 0.002 ^{a,x}
6	0.195 ± 0.002 ^{a,x}	0.193 ± 0.002 ^{a,x}	0.203 ± 0.002 ^{a,x}

Mean (±standard deviation) with the different superscript in the same column (a,b,c,d) and in the same line (x,y,z) for each type of milk are statistically different (Duncan test, $P < 0.05$).

The acids could have been produced in raw milk by naturally present lactic acid bacteria, which could have grown at 10°C. *Lactococci* are known to utilise lactose and some strains can grow well at temperatures of 7°C and below (Kives *et al.* 2005).

No proteolysis was observed at 4 and 7°C during storage of the sterilised milk inoculated with *P. fluorescens*; however, at 10°C, there was a significant increase in proteolysis at the 6th storage day (24%). This result indicates that *P. fluorescens* affected significantly milk protein stability only at 6 days of storage at 10°C. According to Wang and Jayarao (2001), among 55 isolates of *P. fluorescens* from farm bulk tank milk, 80% were proteolytic at 7°C.

The raw milk inoculated with *P. fluorescens* showed significant proteolytic activity at a lower storage temperature (16% proteolysis at 6°C). At 10°C, 12% proteolysis was observed within 2 days of storage, whereas on the 6th storage day the sample coagulated.

The alizarol test indicated positive results only for raw milk at 10°C on the 4th and 6th days of storage. In a similar way, the thermostability test provided positive results only on the 6th storage day of the raw milk at 10°C. At this storage time, the titratable acidity of the raw milk reached 0.310 g/100 mL, which was high enough to destabilise casein, resulting in coagulation.

During descriptive sensory evaluation, the sterilised milk had characteristic smell up to 4 days of storage; however, on the 6th day the samples had a putrid smell at every temperature investigated. In the raw milk, similar results were observed for the samples stored at 4°C and 7°C; whereas at 10°C, the samples had an acidic smell on the 4th day and a putrid smell on the 6th day of storage. Based on these results, *P. fluorescens* by itself or in the presence of the natural milk microbiota promoted a putrid smell on the 6th day of storage.

According to Wang and Jayarao (2001), when the number of *P. fluorescens* is greater than 10^6 , the degrading effect of the proteolytic enzymes could be detected through sensory evaluation. However, such correlation was only found for samples stored at 4°C. At higher storage temperatures, higher counts of psychrotrophic micro-organisms were needed to impart a putrid smell to the samples.

Significant increase in acidity was only observed in the raw milk during storage at 10°C. At this temperature, the samples gave positive results for alizarol (4th day on) and thermostability (6th day on), and the sample coagulated on the 10th day of storage. The milk sample had acid and putrid smells

on the 4th and 6th storage days, respectively. Therefore, raw milk should not be stored at 10°C.

Every sample had a putrid smell on the 6th day of storage at every condition investigated, which suggests that milk samples should be stored for up to 4 days, even at 4°C.

Correlation among parameters investigated

Significant positive correlation ($P < 0.01$) was observed for the raw milk at 10°C between titratable acidity and the total levels of amines (0.9669). These results are in agreement with Gloria (2005); the growth of bacteria in an acidic media favours decarboxylase activity and, consequently, the formation of amines, as a protection mechanism against the adverse pH. Significant correlation was also observed for titratable acidity with proteolytic rate (0.9732) and titratable acidity with undesirable smell (0.9823).

These results indicate that the formation of bioactive amines in the raw milk was associated with the growth of the naturally present microbiota at the higher storage temperature (10°C). Furthermore, the higher titratable acidity favoured proteolytic rate and off flavour development. The naturally present lactic acid micro-organisms in the raw milk were probably responsible for the formation and accumulation of the amines in raw milk. The proteolytic activity of psychrotrophic micro-organisms is more likely to affect amine formation in dairy products, especially those with undergo extent maturation periods compared to fluid milk.

Based on these results, Brazilian authorities should restrict storage of raw milk at bulk tanks in dairy farms or plants of sterilised milk at plants to 4°C for up to 4 days. The temperature of the raw milk should not exceed this temperature in order to prevent the growth of naturally present lactic acid bacteria, which will enhance proteolytic activity, off flavour formation and will favour the formation of bioactive amines.

CONCLUSIONS

Spermine, SPD, PUT, SRT and PHM at low levels were present in the samples immediately after milking. Milk sterilisation caused the formation of CAD. The temperature and time of storage of raw and sterilised milk inoculated with *P. fluorescens* affected the growth of psychrotrophic micro-organisms, with counts proportional to temperature increase (4°C, 7°C and 10°C). Both raw and sterilised milk had a putrid flavour on the 6th day of storage at every temperature. The formation of

bioactive amines was only observed on the 6th storage day at 10°C of the raw milk, suggesting that other naturally present lactic acid micro-organisms in the raw milk were responsible for the formation and accumulation of the amines CAD, HIM and TYM. It is anticipated that higher storage temperature and the simultaneous presence of lactic acid bacteria and psychrotrophic micro-organisms, as well as its proteolytic enzymes, will allow the formation and accumulation of bioactive amines in dairy products, especially those which undergo maturation steps during processing.

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