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ORIGINAL ARTICLE

Bactericidal effect of bovicin HC5 and nisin against *Clostridium tyrobutyricum* isolated from spoiled mango pulp

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Abstract

Aims: To test the effect of bovicin HC5 – a bacteriocin from *Streptococcus bovis* HC5 – against the strains of *Clostridium tyrobutyricum* isolated from canned spoiled mango pulp.

Methods and Results: Bovicin HC5 [40–160 arbitrary unit (AU) ml^{-1}] reduced the specific growth rate and increased the lag phase duration of the bacterial isolates inoculated in brain heart infusion media at 30°C. The inhibitory activity of bovicin HC5 (100 AU ml^{-1}) in mango pulp was bactericidal and more pronounced at acidic conditions. When *C. tyrobutyricum* was inoculated into mango pulp with bovicin HC5, gas production was not observed. Cultures that were successively transferred in the presence of sublethal doses of bovicin HC5 did not become resistant.

Conclusions: The addition of bovicin HC5 to mango pulp might be effective in preventing deterioration by spoilage bacteria.

Significance and Impact of the Study: Bovicin HC5 and nisin have the potential to increase the shelf life of canned fruit pulps.

Introduction

Mango (*Mangifera indica L.*) is an economically important crop fruit that has been widely cultivated in several tropical and subtropical countries (Loeillet 1994; FAO 2000). Mango is one of the most commonly eaten fresh fruits worldwide, and it has also been consumed in the processed form. Food industries can significantly increase the shelf life of mango pulp by heat treatments of the acidified pulp (Azizi and Ranganna 1993). However, spore-forming bacteria, including *Bacillus*, *Clostridium* and *Alicyclobacillus*, can grow even at low pH conditions. These micro-organisms have been associated with the spoilage of several heat-treated acidic foods and often cause off-flavours in fruits juices and pulps during storage (Everis and Betts 2001; Wilkes *et al.* 2000).

Although several studies have been performed regarding the spoilage of processed fruit products, such as orange and apple juices (Pao and Brown 1998), little is known about the microbial biota associated with mango fruits. As endospore-forming bacteria commonly found in the soil can contaminate the mango pulp during processing and handling of the fruit. Drastic heat treatments would be necessary to assure microbiological safety of these fruit products.

Bacteriocins from lactic acid bacteria have been suggested as a potential nonthermal preservation method (Cleveland *et al.* 2001). Bacteriocins are ribosomally synthesized antimicrobial peptides that have activity against other bacteria, either of the same species or across genera (Cotter *et al.* 2005). We have recently demonstrated that bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5 (Mantovani *et al.* 2002), could inhibit the growth and spore germination of *Bacillus cereus* and *Bacillus thuringiensis* isolated from spoiled mango pulp (Carvalho *et al.* 2007). In this study, we demonstrated the bactericidal effect of bovicin HC5 and nisin against the strains of *Clostridium tyrobutyricum* isolated from canned spoiled mango pulp.

Materials and methods

Micro-organisms and growth

Streptococcus bovis HC5 was provided by Dr James B. Russell (Cornell University, Ithaca, NY, USA) and cultured as previously described (Mantovani and Russell 2003). The *C. tyrobutyricum* strains LMA45, LMA63 and LMA72 were previously isolated from spoiled mango pulp obtained from two fruit juice industries located in Minas Gerais State, Brazil (Costa 2006). The isolates were characterized biochemically by Costa (2006) using standard methods and identified by fatty acids methyl ester (FAME) analysis (Sherlock Microbial Identification System, Newark, DE, USA).

Cultures were grown anaerobically in brain heart infusion (BHI) (Acumedia, Baltimore, Maryland, USA) at 30°C and tested for catalase activity and Gram staining. The BHI media was prepared under an oxygen-free CO_2 atmosphere, transferred anaerobically (5 ml) to tubes (18 × 150 mm; Bellco Glass Inc., Vineland, NJ, USA) and sealed with butyl rubber stoppers and aluminum seals. *Lactococcus lactis* ATCC 19435 was cultivated aerobically in de Mann, Rogosa and Sharpe (MRS) broth (de Man *et al.* 1960) at 30°C.

Preparation and activity of bovicin HC5 and nisin

Extracts of bovicin HC5 were prepared as described by Mantovani *et al.* (2002). The activity of bovicin HC5 was determined as described by Hoover and Harlander (1993), using *L. lactis* ATCC 19435 as the indicator organism. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution that showed a zone of inhibition with at least 5 mm diameter.

Nisin solution (1000 U mg^{-1}; Christian Hansen, Denmark, Hǿrsholm) was prepared in phosphate buffer (pH 2·0). Nisin activity was determined as described earlier.

Stability of bovicin HC5 in mango pulp and culture supernatants

Stationary phase cultures of *C. tyrobutyricum* LMA45, LMA63 and LMA72, grown in BHI media without bacteriocin, were centrifuged and the cell pellets were discarded. Bovicin HC5 (100 μl, 320 AU ml^{-1}) was mixed with an equal volume of supernatant and incubated at 37°C for 3 h before the residual activity was tested. Bovicin HC5 treated with trypsin or proteinase K (5 mg ml^{-1}; Sigma, St. Louis, Missouri, USA) and culture supernatants without bacteriocin were used as controls.

To test the stability of bovicin HC5 (320 AU ml^{-1}) in mango pulp, the bacteriocin was added to mango pulp and incubated at room temperature and at 4°C. At 0, 5, 10, 20, 30, 50 and 60 days of incubation, the activity of bovicin HC5 was assayed as described earlier.

Effect of bovicin HC5 and nisin on the growth of the bacterial isolates cultured in liquid media and in mango pulp

Clostridium tyrobutyricum LMA45, LMA63 and LMA72 were inoculated (10^6 CFU ml^{-1}) into BHI broth containing bovicin HC5 (40–160 AU ml^{-1}). Bacterial growth was monitored via changes in optical density (OD) at 600 nm in a Spectronic 20D$^+$ (Thermal Electron, Madison, WI, USA). The specific growth rate, lag phase duration and maximum OD values were determined.

To verify the effect of bovicin HC5 against *C. tyrobutyricum* LMA45, LMA63 and LMA72 in mango pulp, commercial mango pulp was purchased in a local market and diluted twofold with distilled water to reduce viscosity. The solutions (pH adjusted to 4·0, 4·5, 5·0, 5·5, 6·0, 6·5 and 7·0) were heated (121°C, 15 min), gassed with oxygen-free CO_2 and transferred anaerobically (5 ml) to 15-ml vials sealed with butyl rubber stoppers and aluminium seals. Diluted mango pulp containing bovicin HC5 or nisin (100 AU ml^{-1}) were inoculated with 10^6 CFU ml^{-1} of each bacterial isolate. Samples were taken at 0, 12 and 24 h of incubation and the viable cell number was determined. Cultures without bacteriocins were used as control.

Gas production experiments

Clostridium tyrobutyricum LMA45, LMA63 and LMA72 (10^6 CFU ml^{-1}) were inoculated into serum bottles containing anoxic mango pulp (pH 4·0) and bovicin HC5 (100 AU ml^{-1}). Syringes of 10 ml with barrels lubricated with glycerin were used to monitor the volume of gas (ml) produced (Nche *et al.* 1994). The serum bottles were incubated at 30°C and controls were performed without the addition of bacteriocins.

Statistics

Each experiment was performed at least two times in duplicate and the error bars presented indicate the standard deviation of the mean.

Results

Stability of bovicin HC5

The inhibitory activity of bovicin HC5 persisted when the bacteriocin was incubated with culture supernatants from

Table 1 Stability of bovicin HC5 on mango pulp at 4°C and at room temperature (27°C)

Time (days)	Inhibition zone (mm) 4°C	Inhibition zone (mm) Room temperature	Activity (AU ml^{-1}) 4°C	Activity (AU ml^{-1}) Room temperature
0	22 ± 1·41	20 ± 0·71	320	320
5	20 ± 1·01	20 ± 0·61	320	320
10	21 ± 0·60	21 ± 0·75	320	320
20	20 ± 0·10	20 ± 0·55	320	320
30	20 ± 0·15	20 ± 0·35	320	320
50	16 ± 0·50	16 ± 0·72	160	160
60	15 ± 0·80	ND	160	ND
120	13 ± 0·60	ND	80	ND

ND, not determined; AU, activity units.

C. tyrobutyricum (data not shown). A similar result was obtained if bovicin HC5 was treated with proteinase K. The control treatments showed an inhibition zone of 21 mm against the indicator strain, and similar zones were observed for the treatments described earlier (data not shown). Bovicin HC5 remained stable in mango pulp (room temperature or at 4°C) for at least 30 days, and after 50 days, the residual activity was 50% lower than its initial value (Table 1).

Effect of bovicin HC5 and nisin on bacterial isolates cultivated in liquid media and mango pulp

The specific growth rate of *C. tyrobutyricum* LMA45, LMA63 and LMA72 in BHI media were 0·20, 0·17 and 0·22 h^{-1}, respectively (Table 2). Bovicin HC5 (40–160 AU ml^{-1}) inhibited the growth of *C. tyrobutyricum* LMA45 and LMA72 and increased the lag phase duration (≥7 h) of strain LMA63 (Table 1). The specific growth rate of *C. tyrobutyricum* LMA63 was 12% and 18% slower with 40 and 80 AU ml^{-1}, respectively, of bovicin HC5, and the maximal OD of the treated cultures was always less than the control treatments (Table 2). If the bovicin HC5 concentration was increased to 160 AU ml^{-1}, growth was not observed for at least 144 h.

The lag phase duration of *C. tyrobutyricum* LMA45 increased by about 6 h in the presence of 40 AU ml^{-1} of nisin, and growth was not observed for at least 17 h if 80 and 160 AU ml^{-1} were used (Table 2). The growth of *C. tyrobutyricum* LMA63 and LMA72 was not observed when nisin was used (Table 2).

When 10^6 CFU ml^{-1} of *C. tyrobutyricum* LMA45, LMA63 and LMA72 were inoculated into mango pulp with pH ranging from 4·0 to 7·0, an increase in the viable cell number occurred after 12 h of incubation, regardless of the mango pulp pH (Fig. 1). The strains reached a viable cell number of 10^8 CFU ml^{-1} at all the pH values tested (Fig. 1), except *C. tyrobutyricum* LMA63 (10^7 CFU ml^{-1}) grown at pH 5·5 and 6·0 (Fig. 1b).

Bovicin HC5 decreased the viable cell number of *C. tyrobutyricum* LMA45 below the detection level (10^2 CFU ml^{-1}) for all pH values tested (Fig. 1a). The viability of *C. tyrobutyricum* LMA63 and LMA72 reduced as the mango pulp pH decreased, and at least a three log cycle difference was seen in the pH range of 4·0 to 5·5 compared with control treatments without bacteriocin (Fig. 1b,c). When nisin was used (100 AU ml^{-1}), the viable cell number of *C. tyrobutyricum* LMA45 decreased three to five log cycles (Fig. 1a). The viability of *C. tyrobutyricum* LMA63 and LMA72 was below the detection level at all pH values in mango pulp containing nisin (Fig. 1b,c).

Table 2 Effect of bovicin HC5 and nisin on the growth of *Clostridium tyrobutyricum* LMA45, LMA63 and LMA72

Micro-organism	Bacteriocin concentration (AU ml^{-1})	Specific growth rate (h^{-1}) Bovicin HC5	Specific growth rate (h^{-1}) Nisin	Lag phase duration (h) Bovicin HC5	Lag phase duration (h) Nisin	Maximal optical density (600 nm) Bovicin HC5	Maximal optical density (600 nm) Nisin
C. tyrobutyricum LMA45	0	0·20	0·20	6·00	6	0·92	0·92
	40	–	0·12	>144	12	–	0·96
	80	–	ND	>144	>17	–	0·98
	160	–	ND	>144	>17	–	0·91
C. tyrobutyricum LMA63	0	0·17	0·17	6·00	6	1·00	1·00
	40	0·15	–	13	>144	0·64	–
	80	0·14	–	14	>144	0·55	–
	160	–	–	>144	>144	–	–
C. tyrobutyricum LMA72	0	0·22	0·22	3·00	3	1·05	1·05
	40	–	–	>144	>144	–	–
	80	–	–	>144	>144	–	–
	160	–	–	>144	>144	–	–

–, no growth; ND, not determined; AU, activity units.

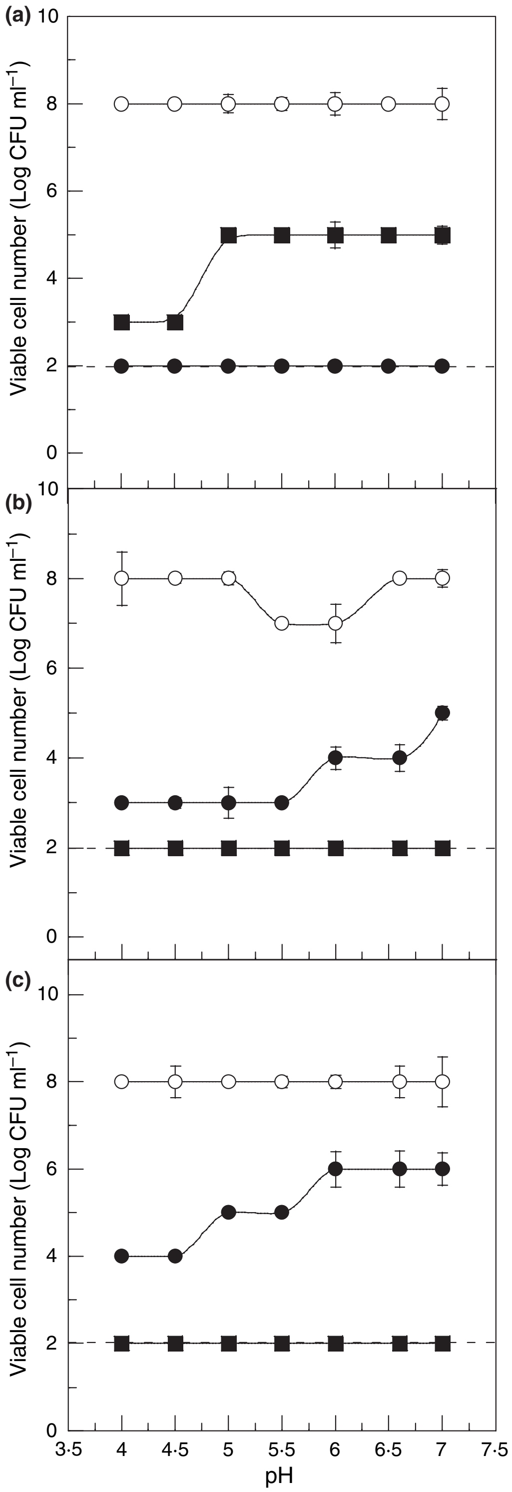

Figure 1 Viable cell number of *Clostridium tyrobutyricum* LMA45 (a), LMA63 (b) and LMA72 (c) inoculated (10^6 CFU ml^{-1}) into mango pulp at different pH values. Bovicin HC5 (closed circles) and nisin (closed squares) were added to the mango pulp at 100 AU ml^{-1}. Control treatments without bacteriocin are also shown (open circles). The dotted line shows the detection limit of the enumeration.

After 24 h of incubation, both bovicin HC5 and nisin reduced the viable cell number of all strains below the detection level at all the pH tested (results not shown). These results indicated that the effect of bovicin HC5 and nisin was bactericidal and the antibacterial activity was more pronounced under acidic conditions.

Effect of bovicin HC5 on gas production

Gas production initiated after 4 h of incubation in mango pulp (pH 4·0) for *C. tyrobutyricum* LMA45 and LMA63 and after 12 h for *C. tyrobutyricum* LMA72 (Fig. 2). The maximal gas production was 18·6, 12·6 and 22·0 ml by *C. tyrobutyricum* LMA45, LMA63 and LMA72, respectively (Fig. 2). If bovicin HC5 was added to the mango pulp, gas production did not occur even after 10 days of incubation (data not shown).

Development of resistance

Clostridium tyrobutyricum LMA45, LMA63 and LMA72 were transferred in BHI media for approximately 40 generations with sublethal doses of bovicin HC5 (20 AU ml^{-1}) and tested for the development of resistance. The treated cultures could not resist the doses of bovicin HC5 that were previously inhibitory, and the *C. tyrobutyricum* LMA63 had an increased lag phase (≥36 h) compared with untreated cultures that were exposed to similar doses of bovicin HC5 (data not shown). Bovicin HC5 at 160 AU ml^{-1} inhibited the growth (144 h of incubation) of all the isolates tested. Similar results were obtained when nisin was used (data not shown).

Discussion

Clostridium spp. are commonly found in soil and can contaminate fruits and vegetables. Because they form endospores, these bacteria can survive several processing methods used in the food industry (Setlow and Johnson 2001). These endospores can germinate after the heat treatment, which can either lead to deterioration of the food product or result in the production of toxins that are harmful to consumers. To control these micro-organisms, it has been suggested the use of bacteriocins in foods as an additional hurdle (Cleveland *et al.* 2001; Cotter *et al.* 2005). In this study, we showed the effect of bovicin HC5 and nisin against the strains of *C. tyrobutyricum* isolated from spoiled mango pulp that had been heat treated.

The inhibition of growth and elimination of pathogenic or spoilage micro-organisms by bacteriocins during processing and storage of foods result from the interactions of bacteriocins with food matrices and micro-organisms

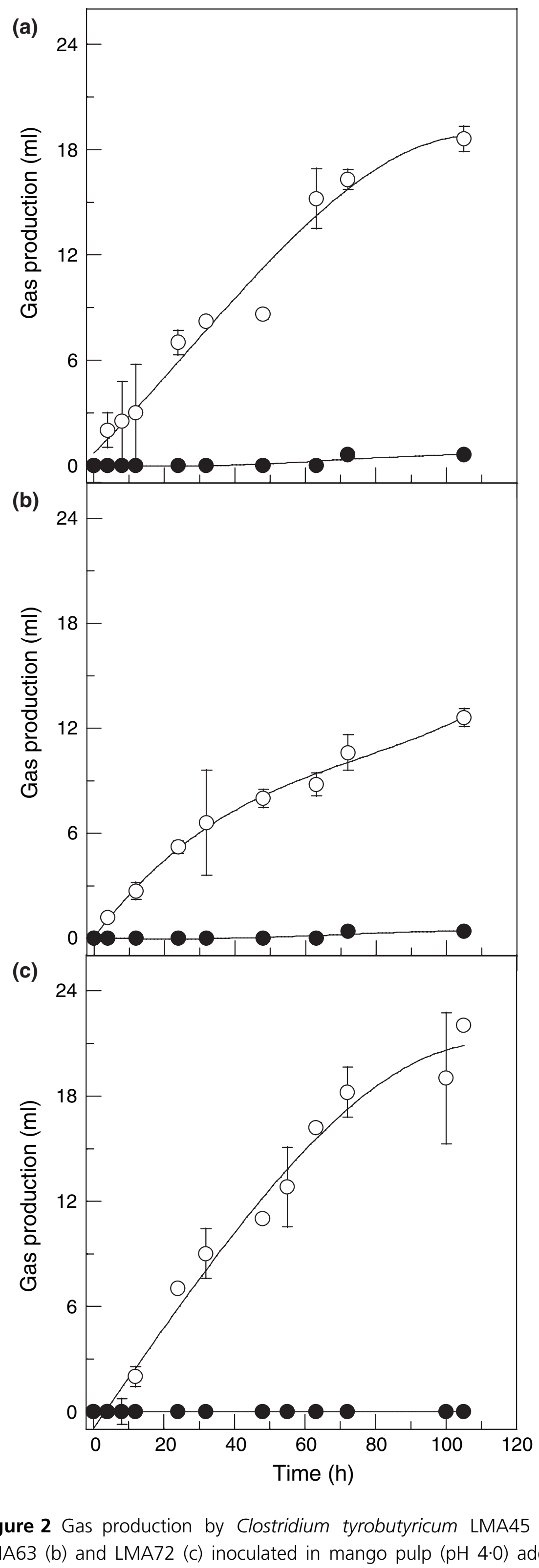

Figure 2 Gas production by *Clostridium tyrobutyricum* LMA45 (a), LMA63 (b) and LMA72 (c) inoculated in mango pulp (pH 4·0) added with bovicin HC5 (closed circles) at 100 AU ml^{-1}. Gas production (ml) was monitored for 105 h of incubation at 30°C. Controls without bacteriocin are also shown (open circles).

(Gänzle *et al.* 1999). However, the application of bacteriocins in foods can be limited by properties, such as the charge and solubility of the peptide, the interaction of bacteriocins with food components, food pH and inactivation by proteases (Gänzle *et al.* 1999).

Bovicin HC5 was stable in the supernatants from *C. tyrobutyricum* strains, and similar results were obtained when bovicin HC5 was incubated with mango pulp. Based on these results, bovicin HC5 does not appear to interact with the components of mango pulp or to be inactivated by supernatants from the cultures of *C. tyrobutyricum* used in this study. Bovicin HC5 decreased the growth rate and maximal OD and increased lag phase duration of spoilage bacteria isolated from mango pulp. These results were comparable with the inhibitory effect of nisin. Because bovicin HC5 resembles the lantibiotics (Mantovani *et al.* 2002), the effect of both bacteriocins on the target bacteria tested could be similar.

Bovicin HC5 was bactericidal against the vegetative cells of *Clostridium* spp. inoculated in mango pulp, and these effects were more pronounced under acidic conditions. These observations agree with the results of Houlihan *et al.* (2004), who demonstrated that the activity of bovicin HC5 was pH dependent. Because bovicin HC5 inhibited the strains of *C. tyrobutyricum* LMA45, LMA63 and LMA72 even when the initial inoculum was as high as 10^6 CFU ml^{-1}, it appears that bovicin HC5 could prevent the spoilage of mango pulp caused by clostridia.

Food spoilage caused by the species of *Clostridium* often results in gas production (Azizi and Ranganna 1993). Our results indicated that bovicin HC5 inhibited gas production by *C. tyrobutyricum* inoculated in mango pulp (pH 4·0) during a period of incubation of approximately 10 days. Because the strains of *C. tyrobutyricum* used in this study did not produce ammonia in mango pulp and complex media, these strains are probably saccharolytic. Saccharolytic clostridia usually produce CO_2 and H_2 as end products of the carbohydrate fermentation, and ATP is obtained by substrate-level phosphorylation (Doelle 1975). Because ion efflux (e.g. K^+ loss) by pore-forming peptides can increase ATPase activity (Carneiro de Melo *et al.* 1996), the insertion of bacteriocins in the cell membrane of *C. tyrobutyricum* could deplete the ATP pool and impair carbohydrate fermentation.

The application of bacteriocins in food preservation can also be affected by adaptation or selection of resistant mutants in sensitive populations (Mazzotta *et al.* 1997; Cotter *et al.* 2005). Several studies have demonstrated that previously sensitive cells can become resistant to bacteriocins, such as nisin and pediocin PA-1 (Crandall and Montville 1998; Mantovani and Russell 2001). Our results showed that cells transferred with bovicin HC5 did not grow in the presence of previously inhibitory concentrations of the bacteriocin. These results indicated that the resistance to bovicin HC5 is not readily acquired by the isolates tested in this study.

Although many bacteriocins have been purified and characterized, to date, the only commercially produced bacteriocins are nisin, and to a lesser extent, pediocin PA-1 (Cotter *et al.* 2005). Because nisin is shown to be ineffective in some food matrices (e.g. meat), and considering the potential emergence of nisin-resistant populations, it seems attractive to explore the use of other bacteriocins to prevent microbial growth in food products (Deegan *et al.* 2006). Some *S. bovis* strains can cause infection in humans, but bovine and human *S. bovis* isolates seem genetically and physiologically distinct (Jarvis *et al.* 2000; Kurtovic *et al.* 2003). More studies are needed to see if bovicin HC5 has any cytotoxic or allergenic effects on mammalian cells.

Based on our results, it appears that bovicin HC5 was effective against spoilage *C. tyrobutyricun* isolated from mango pulp, and development of resistance was not observed. These observations suggest that bovicin HC5 could prevent the growth of *Clostridium* strains in acidified and heat-treated mango pulp. However, more studies are needed to find if this peptide also affects the germination or survival of spores from spoilage bacteria found in fruit pulps. Preliminary results indicated that bovicin HC5 is very effective in reducing the survival of spores produced by the thermoacidophilic spore former *Alicyclobacillus acidoterrestris*, another important acid-spoilage bacteria.

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