Growth of Selected Food Spoilage and Pathogenic Bacteria under Modified Atmosphere

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The effects of carbon dioxide on the growth of food spoilage and pathogenic bacteria, *Pseudomonas aeruginosa*, *P. fluorescens*, *Escherichia coli*, *Salmonella typhimurium*, *Vibrio parahaemolyticus*, nonagglutinating (NAG) vibrio, *Staphylococcus aureus*, *Bacillus cereus*, *B. subtilis*, *B. macerans*, *B. licheniformis*, *Weissella (Lactobacillus) viridescens*, *Enterococcus faecalis*, and *Clostridium perfringens* were investigated on solid media at 30°C. Growth of the bacteria on the surface of Plate Count Agar (PCA) packaged with different concentrations of carbon dioxide (0, 20, 40, 60, 80, 100% in N₂ gas) was evaluated after 24 h incubation at 30°C. Growth of *P. aeruginosa*, *P. fluorescens*, *E. coli*, *S. typhimurium*, *V. parahaemolyticus*, NAG vibrio, and *S. aureus* was inhibited by carbon dioxide in proportion to the increase in the ratio of carbon dioxide in N₂ gas mixtures. Particularly, a significant growth inhibition was observed for two species of *Pseudomonas*, *S. aureus*, and NAG vibrio. Growth of 4 species of *Bacillus* (*B. cereus*, *B. subtilis*, *B. macerans*, *B. licheniformis*) was not inhibited by carbon dioxide. Growth of 2 species of lactic acid bacteria (*W. viridescens*, *E. faecalis*) and a species of obligate anaerobic clostridia (*C. perfringens*) was stimulated by CO₂.

Key words: modified atmosphere packaging, food pathogens, food spoilage bacteria, carbon dioxide

The sales of fish fillets in supermarkets instead of those of whole or semi-dressed fish traditionally processed in small local retail shops are currently increasing in commercial markets of fisheries products in Japan. Also, consumers are currently eating more fresh fish instead of frozen fish. To meet the demands for longer shelf life of those products, modified atmosphere (MA) packaging methods could be one choice.^{1–6)} Our previous studies have demonstrated that CO₂ storage retarded the growth of aerobic spoilage microorganisms^{3,7,8)} and reduced the potential health hazard caused by several food pathogens^{9,10)} in fish packaged with carbon dioxide controlled atmospheres.

The inhibitory effect of CO₂ for the mixed spoilage populations of microorganisms found in food and the behavior of specific pathogens in MA food in comparison with air packagings have widely been studied; see e.g. reviews by Farber¹¹⁾ and Kimura and Fujii.¹²⁾ However, up to now, relatively few comparative studies on pure cultures have been performed.^{13–15)} Some earlier investigations were done in liquid media using 96-tissue culture plates¹³⁾ or fermenters flushed continuously with a gas mixture.¹⁴⁾ However, it is not possible to extrapolate the data from a liquid culture to a food system, because different results between liquid experiment and food samples could be obtained due to the solid-gas interface. The agar media system is an attempt to model the growth of food pathogens on food under MA storage.¹⁶⁾ Recently the effects of CO₂ on the growth of some food borne pathogens and spoilage bacteria in the

agar media system under MA mixed with O₂ have been studied.¹⁵⁾ However, it is still not possible from the literature to conclude which pathogens are more and which are less resistant to carbon dioxide under anaerobic condition, i.e., MA with the mixture of CO₂ and N₂. In particular, the behavior of food pathogens and other food related bacteria under abusive temperature conditions is not clear at present. Temperature abuse is common throughout the distribution and retail markets. Particularly, the proper control of temperature during consumer handling is quite difficult, thus the behavior of food related bacteria under higher temperature such as 30°C should be studied.

In the present study, our purpose was to test the effect of CO₂ gas on the anaerobic growth of selected food borne bacteria in the agar media system, a solid surface system which simulated conditions on the surface of packaged foods stored under abusive temperature conditions.

Materials and Methods

Cultures

Food related bacteria used were *Pseudomonas fluorescens* (IFO 14160), *P. aeruginosa* (IFO 12689), *Escherichia coli* (IFO 3806), *Salmonella typhimurium* (IFO 13245), *Vibrio parahaemolyticus* (IFO 12711), NAG vibrio (SUF 19921), *Bacillus subtilis* (IFO 13719), *B. macerans* (IFO 3490), *B. cereus* (IFO 13494), *B. licheniformis* (IFO 12107), *Staphylococcus aureus* (IFO 37611), *Weissella (Lactobacillus) viridescens* (IFO 3949), *Enterococcus faeca-*

lis (IFO 12964), and *Clostridium perfringens* (BBC 2401).

Experimental Design

P. aeruginosa, *P. fluorescens*, *E. coli*, *S. typhimurium*, *S. aureus*, *B. cereus*, *B. subtilis*, *B. macerans*, *B. licheniformis*, *W. viridescens* and *E. faecalis* were individually incubated on Plate Count Agar slant (Nissui) at 30°C for 24 h and loopfuls of them were suspended in sterile saline water (0.85% NaCl) to an OD₆₅₀ of approximately 0.8 to obtain inoculum suspensions for experiments. *V. parahaemolyticus* and NAG vibrio were incubated on PCA prepared with a mixture of distilled water and aged sea water (final salinity 2.5%) and treated the same as described above. *C. perfringens* was incubated in cooked meat medium (Nissui) under anaerobic condition (BBL GasPak system) at 30°C for 24 h and portions of the culture were diluted with sterile salt water to produce an inoculum suspension. The inoculum suspension was inoculated onto the surface of PCA in petri dishes.

Immediately after inoculation, the petri dishes were packaged in 300 × 200 mm gas-impermeable laminated film bags (K-nylon 15 µm/polyethylene 65 µm) with two dishes in each package. The gas transmission rate of the film was 0.1 g/m²/24 h/atm for CO₂ and 0.03 g/m²/24 h/atm for O₂. The desired gas mixture was attained by blending CO₂ and N₂ using a Crown gas regulator (MXF-1, Yutaka Engineering Co., Ltd.). A vacuum was evacuated to a negative pressure of 760 mm Hg on each tray individually, and the selected gas mixtures (air, N₂ 100%, CO₂ 20%, 40%, 60%, 80%, 100% in N₂) were introduced and then heat sealed using a Model V-400G gas packaging machine (Tosei Electrics Co., Ltd., Japan). In the CO₂ gas atmosphere, the level of O₂ content was less than 0.1% and the range of the CO₂ content was 40 ± 2%, as determined by gas partition chromatography (GC-3BT, Shimadzu) fitted with a thermal conductivity detector.

Growth Measurement

Petri dishes were removed after 24 h incubation at 30°C and bacteria grown on the surface of the agar plates were washed with 5 ml of sterile saline water 3 times (total 15 ml), and combined into a single suspension. Bacterial growth was determined by optical density of the rinsed suspensions. Duplicates were used for all experiments. Relative growth values were given as % optical density of the control (CO₂ 0%: N₂ 100%).

Results

The effects of various percentages of CO₂-in-N₂ on the growth of selected food related bacteria were compared after 24 h incubation on the surface of PCA agar at 30°C. The growth with air was also measured for comparison. Typical results are shown in Fig. 1. For all the species except for *C. perfringens*, the growth yield under MA (the mixture of CO₂ and N₂) was significantly lower than those obtained with air as expected; i.e., the percent growth of air was 1,373% for *P. fluorescens*, 1,347% for *B. cereus*, 254% for *S. aureus*, 703% for *V. parahaemolyticus*, 680% for *E. faecalis*, and 0% for *C. perfringens*.

Carbon dioxide at all the concentrations in the head space gas (20, 40, 60, 80, 100%) used was found to inhibit

significantly the growth of *P. fluorescens* and *S. aureus* at 30°C. The growth yield under CO₂ atmosphere appeared to decrease as the concentration of CO₂ became higher. For these 2 species, the growth inhibition was still observed with 40% CO₂, which is the proper concentration of CO₂ generally suggested for fish packages. Above this concentration some stimulative effects on the sensory tests would appear when eaten immediately after opening the packages.³⁾ Less significant growth inhibition was observed for *V. parahaemolyticus*, for which the growth inhibition was observed only with higher CO₂ concentrations such as 80% and 100%. No growth inhibition was found for *B. cereus*. The growth yield was virtually the same with any CO₂ concentration, except that slightly stimulated growth was observed at lower CO₂ concentrations (CO₂ 20% and 40%). The growth of *E. faecalis* and *C. perfringens* were promoted with the mixture of CO₂ in the head space gas. Particularly, the growth promotion with 20 and 40% of CO₂ for *C. perfringens* was obvious, since no apparent growth of this organism was obtained with N₂ under the experimental conditions.

From the above results, it was suggested that there is a clear difference in the effect of CO₂ on bacterial growth depending on bacterial species. To analyze this further, the experiments were extended with other food related bacteria; i.e., *P. aeruginosa*, *E. coli*, *S. typhimurium*, NAG vibrio, *B. subtilis*, *B. macerans*, *B. licheniformis*, and *W. viridescens*. The same procedures were used. Representative data are shown in Table 1. The overall data suggested approximately the same effect depending on bacterial groups; i.e., aerobic spoilage bacteria (*P. aeruginosa*) was significantly inhibited, whereas Gram-negative facultative anaerobic pathogens (*E. coli*, *S. typhimurium*) were less inhibited. Gram-positive *Bacillus* (*B. subtilis*, *B. macerans*, *B. licheniformis*) and lactic acid bacteria (*W. viridescens*) were not at all inhibited or were even slightly promoted. Among Gram-negative facultative anaerobic pathogens, an exception was NAG vibrio, whose growth was significantly inhibited.

Discussion

The data presented here indicate that the growth of two strains of *Pseudomonas* and a strain of *S. aureus* and NAG vibrio were inhibited with CO₂ even at 30°C. In the CO₂ gas atmosphere packaging system used in this and our previous studies, the level of O₂ content was less than 0.1%, as determined by gas chromatography.⁸⁾ Although *Pseudomonas* is supposed to be a strictly aerobic bacteria, it is well known that these bacteria can grow with the small amount of residual O₂ present in MAP.¹⁷⁾ Eyles *et al.*¹⁶⁾ reported that *Pseudomonas* could grow on agar medium in petri dishes packaged with 100% N₂ gas atmosphere with a similar method to that of this study. They have shown that the growth of two strains of psychrotrophic *Pseudomonas* (*P. fluorescens* and *P. putida*) was significantly inhibited at as low as 20% CO₂ when storage temperatures were 15°C. Our data suggest that CO₂ storage could be useful for the prevention of growth of those bacteria even under outstandingly abusive temperature conditions, such as 30°C. At present, it is not clear why NAG vibrio was so sensitive to CO₂ while *V. parahaemolyticus*, a

Fig. 1. Growth of *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Bacillus cereus*, *Enterococcus faecalis*, and *Clostridium perfringens* on the agar plate surface at 30°C for 24 h packaged with different CO₂ concentration (balance N₂).

Relative growth values are given as % optical density of control (CO₂ 0%:N₂ 100%). Means in duplicate runs $\pm$ standard deviation are plotted. For *C. perfringens*, no apparent growth was observed in CO₂ 0%, thus the growth in CO₂ 20% was calculated as 100%. The relative growth (%) in air is also shown as the figures (Air=) on the graphs.

Table 1. The effect of various concentrations of carbon dioxide on growth of food related bacteria after 24 h at 30°C

Bacteria	CO ₂ concentration (% in N ₂)						Air
	0	20	40	60	80	100	
<i>Pseudomonas</i>							
<i>P. aeruginosa</i>	100	64 ^a	58	74	36	0	2300
<i>Vibrio</i>							
NAG vibrio	100	80	20	12	15	2	449
<i>Salmonella</i>							
<i>S. typhimurium</i>	100	83	74	68	68	59	638
<i>Escherichia</i>							
<i>E. coli</i>	100	106	101	92	87	84	457
<i>Bacillus</i>							
<i>B. subtilis</i>	100	123	129	139	133	140	1708
<i>B. macerans</i>	100	113	131	95	119	86	962
<i>B. licheniformis</i>	100	120	151	152	85	54	856
<i>Weissella</i> (<i>Lactobacillus</i>)							
<i>W. viridescens</i>	100	507	293	327	195	208	673

^a % growth of the control (CO₂ 0%, N₂ 100%) as measured by optical density of rinsing buffer. Values were the means in duplicate runs.

physiologically similar vibrio group, was not. Also, the data presented here indicate that *S. aureus* is extremely sensitive to CO₂ even at an extremely abusive temperature. The literature revealed that Gram-positive bacteria in general are considered to be more resistant to CO₂ than Gram-negative ones. The conclusion was drawn from general observations on the dominating microflora of both meat and fish products in CO₂ MAP food.^{11,18,19} The dominant groups in the food change from Gram-negative to slow-growing Gram-positive bacteria. Yet, the mechanism of CO₂ inhibition is not well elucidated, and there is no theoretical background for this general assumption. In fact, several Gram-positive bacteria have been demonstrated to be sensitive for CO₂-growth inhibition.¹¹ Our data also supports the hypothesis that CO₂-sensitivity cannot be explained by bacterial groupings in terms of Gram-positive or Gram-negative.

We observed only a slight biostatic effect of CO₂ for *V. parahaemolyticus*, 2 species of enteric groups (*E. coli*, *S. typhimurium*) and almost no biostatic effect for 4 species of *Bacillus* at 30°C. Although most of the studies to date indicate that *B. cereus* is relatively sensitive to CO₂,¹¹ the data presented here indicate that there is no inhibitory effect of CO₂ for all the *Bacillus* strains including *B. cereus* at 30°C for 24 h. Generally, decreased biostatic activity of CO₂ at higher temperatures has been suggested.²⁰ Previous studies on chilled-stored CO₂-MAP revealed that the biostatic activity of CO₂ was most effective at lower temperatures such as below 10–15°C.^{3,11,20} Decreased inhibition by CO₂ at higher temperature was explained as being due to decreased solubility of CO₂ in the food.²⁰ The lack of inhibitory effect against *V. parahaemolyticus* at this temperature is of significance for food safety, since food poisoning incidents caused by this organism under temperature abuse by consumers seem to be high in Japan.

The growth of lactic acid bacteria (*E. faecalis*, *W. viridescens*) was even stimulated with CO₂ under our experimental conditions. The MAP with high CO₂ concentrations under chilled conditions for a relatively long time

generally change the dominating microflora of both meat and fish products into lactic acid bacteria,²¹ although a short storage period (2 to 3 d at refrigeration temperature) of them does not cause such a change.^{7,22} Thus lactic bacteria is generally considered to be a CO₂ tolerant bacteria. The data presented in this study at abusive temperature conditions confirmed the general resistance of lactic acid bacteria.

An important finding in the present study was that the growth of a strain of *Clostridium perfringens* was significantly promoted with CO₂ at 30°C. This suggests that the usage of CO₂ would bring a potential hazard for the growth of anaerobic clostridia under obviously abusive temperature conditions such as 30°C. Modified atmosphere (CO₂) studies on the growth of *C. perfringens* seem inconclusive. Enfors and Molin²³ found that the germination of spores of *C. perfringens* could be stimulated under 1 atm CO₂ conditions compared with 1 atm N₂. However, Mead²⁴ reported a longer lag phase in the growth of this organism under 1 atm CO₂ compared to 1 atm N₂ at 20°C. Hintlian and Hotchkiss²⁵ reported that *C. perfringens* could grow well under a modified atmosphere (75% CO₂, 15% N₂, 10% O₂) at a higher temperature of 26.7°C in MA packaged beef while a combination of the MA and low temperature (12.8°C) inhibited the growth of the organisms. In our previous study,¹⁰ we have already presented data showing that the growth of *C. perfringens* could be stimulated on CO₂-MAP fish fillet (Jack mackerel) compared with N₂-MAP fish fillet stored under obviously abusive temperature (30°C) even for a relatively short period (4 h). The present data obtained in the agar plate system strongly supported our previous observation in MAP fish studies, indicating the significance of strict temperature control in CO₂-MAP. Further studies on clostridia including *C. botulinum* type E is needed in this respect.

It should be mentioned that the growth of test organisms in this study may be influenced by the combined effect of CO₂ and a fall in pH, since the pH of culture medium was uncontrolled during growth in this study. Hydration of CO₂ to carbonic acid is estimated to be 1 to 2%²⁶ and carbonic acid dissociates to form bicarbonate and hydrogen ions at pH > 6.0, which likely causes a pH decrease. The pH of culture medium was 7.0 at the start of the experiments and that of the uninoculated medium stored under 20%, 40%, 60%, 80%, and 100% CO₂ dropped to 6.2, 6.0, 5.8, 5.6 and 5.6 respectively after 24 h at 30°C (data not shown). Hence some of the observed growth inhibition with high concentration of CO₂ such as 100% CO₂ might be overestimated in this study, although the minimal pH decrease (generally 0.5–1.0) by the hydration of CO₂ would not cause a significant biostatic activity; e.g., *E. coli* can grow with little difference in generation time over a pH range of 5.5 to 8.5.²⁷ Apparently, these aspects should be studied more carefully, including the study on the mechanism on the different sensitivity to CO₂ depending on the bacterial species as described above. These aspects are currently being investigated.

Whereas the agar plate system used in this study includes some experimental artifacts as stated above, we believe that the present results reflect a general assessment of the behavior of food related bacteria on food under MA under abusive temperature condition.

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