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Effect of modified atmosphere packaging on the TVB/TMA-producing microflora of cod fillets

J. Debevere*, G. Boskou

Department of Food Technology and Nutrition, Faculty of Agricultural and Applied Biological Sciences, University of Ghent, Coupure Links 653, 9000 Ghent, Belgium

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Abstract

Cod fillets (*Gadus morhua*) were packed under modified atmospheres, with four different gas compositions (60% CO_2-10% O_2-30% N_2, 60% CO_2-20% O_2-20% N_2, 60% CO_2-30% O_2-10% N_2, 60% CO_2-40% O_2), and stored at 6°C. Plate counts were carried out after 3, 4, 5, 6 and 7 days, to follow the growth of aerobic and anaerobic bacteria, lactic acid bacteria, H_2S-producing bacteria and *Enterobacteriaceae*. The production of total volatile bases (TVB) and trimethylamine (TMA), and the changes in pH of the fillets were measured. Modified atmosphere packaging (MAP) had in general an inhibitory effect on the growth of the microflora but limited inhibition of the production of TVB and TMA. Despite the fact that increased oxygen proportions in the atmosphere contributed in a slightly lower production of TMA, all the samples had a TVB and TMA content high enough to be considered as spoiled after 4 days' storage at 6°C. A total aerobic plate count at 25°C of a 10^6 cfu/g, combined with the presence of only a 10^3 cfu/g of H_2S-producing bacteria, which are normally considered as TMAO-reducing organisms in fish, cannot explain the strong increase in TMA. A high cell concentration of more than 10^8 cfu/g of *Shewanella putrefaciens* is required for production of a TMA level normally found in spoiled fish. This suggests that there could be another type of bacterium in fish, not involved in the spoilage of unpacked fish, which is resistant to 60% CO_2, is not H_2S-producing, and shows a high TMAO-reducing capacity. This bacterium could be *Photobacterium phosphoreum*.

* Corresponding author. Tel: + 32 9 2646177; fax + 32 9 2255510.

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1. Introduction

Following early reports that carbon dioxide enriched atmosphere prolongs the market life of fish (Coyne, 1932; Shewan, 1950), considerable research has been done to show that modified atmosphere packaging (MAP) extends the shelf-life of fish and fishery products (Statham, 1984; Farber et al., 1990; Stammen et al., 1990). Carbon dioxide is especially effective in inhibiting the typical H_2S-producing spoilage microflora (Jensen et al., 1980)

The microbial flora of fish from sea water consists of Gram-negative, psychrotrophic, aerobic or facultative anaerobic bacteria: *Pseudomonas*, *Alteromonas*, *Shewanella*, *Moraxella*, *Acinetobacter*, *Flavobacterium*, *Cytophaga* and *Vibrio* (Debevere and Voets, 1974; Gram et al., 1987; Huss, 1988). The most active of the spoilage bacteria are *Shewanella putrefaciens* (previously known as *Alteromonas putrefaciens*) and certain *Pseudomonas*, *Vibrio* and *Aeromonas* spp. (Shewan, 1977; Donald and Gibson, 1992). These bacteria are classified as H_2S-producing bacteria that can be enumerated on the Iron Agar 3 selective media of Gram (Gram et al., 1987). Most of these bacteria are facultative anaerobic microorganisms. When oxygen levels are depleted, TMAO which is a characteristic part of the non protein nitrogen fraction (NPN) of the fish muscle (Huss, 1988), serves as a terminal electron acceptor for anaerobic respiration (Easter et al., 1983) and is reduced to trimethylamine (TMA).

The total volatile bases fraction (TVB) includes ammonia, monoethylamine, dimethylamine along with trimethylamine (TMA). TVB is part of the NPN fraction of the fish muscle as well. The residual by the subtraction of TMA from TVB is called formalin-bound volatile nitrogen (FBVN), according to the Dyer method for TMA determination (Dyer, 1945). This fraction of TVB can increase slightly during storage due to some reactions of autolysis and deamination. TMA and TVB are considered responsible for unpleasant 'fishy' odor.

Fresh cod normally has less than 20 mg N/100 g TVB and 3 mg N/100 g TMA. When the level of TVB and TMA exceeds 35 mg N/100 g and 15 mg N/100 g respectively, the fish is considered spoiled (Connell and Shewan, 1980; Huss, 1988).

In this study modified atmosphere packaging was applied on fresh cod. Four different gas mixtures were used. In all four cases the proportion of CO_2 was at the relatively high level of 60%. The proportion of oxygen was set at 10, 20, 30 and 40%, respectively. The objective was to examine the inhibitory effects of CO_2 on the microflora of the cod and to investigate the production of TVB-TMA in relation to a variable availability of oxygen.

2. Materials and methods

2.1. Sample preparation

Fresh cod fillets (*Gadus morhua*) were divided in portions of 100 g each, and 3 portions were placed in polypropylene (PP) trays (15 cm × 10 cm × 5 cm). The trays were then introduced in 25 cm × 35 cm plastic bags with high oxygen barrier (Sidamil, UCB Transpac, Belgium). The plastic bags are made out of PVDC, laminated with polyester, and have a gas-permeability of 6 cc/m^2/24 h for O_2, 2 cc/m^2/24 h for N_2 and 15 cc/m^2/24 h for CO_2 at 25°C and 100% RH. The bags were filled and sealed with an A-300, Downers grave (CVP Systems Inc.), packaging set. The following mixtures were used: 60% CO_2, 10% O_2, 30% N_2 (**G613**), 60% CO_2, 20% O_2, 20% N_2 (**G622**), 60% CO_2, 30% O_2, 10% N_2 (**G631**), 60% CO_2, 40% O_2, 0% N_2 (**G640**). One volume of modified atmosphere (300 ml per 300 g of fish) was added. Five samples of each gas mixture were stored at 6°C and 95% RH. The day the samples were prepared was considered as day 0. At days 0, 3, 4, 5, 6, and 7 chemical and microbiological analysis were performed.

2.2. Microbiological analysis

Thirty g of fish sample were collected aseptically in a stomacher bag and diluted ten times with sterile physiological saline-peptone solution (PS, 0.85% NaCl, 0.1% peptone, pH 7). After homogenizing in a Colworth Stomacher 400 (Seward Laboratory, London, England, UK), a series of tenfold dilutions was made in PS for microbiological analysis.

Total number of bacteria was determined as poor plate counts in PCA (Plate Count Agar, Oxoid CM463) after incubating for 5 days at 25°C. The number of anaerobic bacteria was determined as poor plate counts in PCA but incubation was carried out in an anaerobic jar, for 5 days at 25°C. Lactic acid bacteria were determined as poor plate counts in MRS agar (Oxoid CM361) with a cover layer after 5 days at 30°C. *Enterobacteriaceae* were determined as poor plate counts in Violet Red Bile Glucose Agar (VRBGA, Oxoid CM485) after 24 h incubation at 37°C. Hydrogen sulfide-producing bacteria were counted as black colonies of poor plate counts in Iron Agar Lyngby (Oxoid CM867) with cover layer after 5 days at 30°C.

2.3. Chemical analysis

Before opening, the gas composition of the bags was determined with a Servomex gas analyzer (Food Package Analyzer, Series 1400).

Immediately after opening, 10 g from each bag was aseptically collected and placed in the inner flask of the Antonacopoulos steam-distillation apparatus (Antonacopoulos, 1960). Steam distillation for the collection of the TVB was carried out according to the method of Lüecke and Geidel (Vyncke, 1969). The distillate was titrated by 0.1 N H_2SO_4 (p.a.) with methyl-red as indicator. Before the

addition of the indicator in the distillate, 2 ml were collected for trimethylamine analysis. TMA was determined using the Dyer spectrophotometric method (Dyer, 1945). The absorbance at 410 nm was measured with a Bausch and Lomb, Spectronic 1001 spectrophotometer.

Next, 100 g of each bag was blended separately and pH was measured in the blended fish muscle with an Ingold sharp point electrode connected to a Knick, Multi Climatic, Microprocessor pH-meter.

3. Results

3.1. Microbiological analysis

Fig. 1 shows a poor increase in the number of the total aerobic bacteria for all of the gas atmospheres applied. Storage at low temperature (6°C) in combination with the presence of 60% CO_2 inhibits bacterial growth. No difference can be observed between the total aerobic counts for the different gas mixtures.

The number of anaerobic bacteria did not increase during the first 3 days (Fig. 1), which can be attributed to the inhibitory effect of CO_2 at low temperature (6°). From the fourth day on, an exponential increase of the anaerobic count was observed and subsequently it reached the same levels as for the total

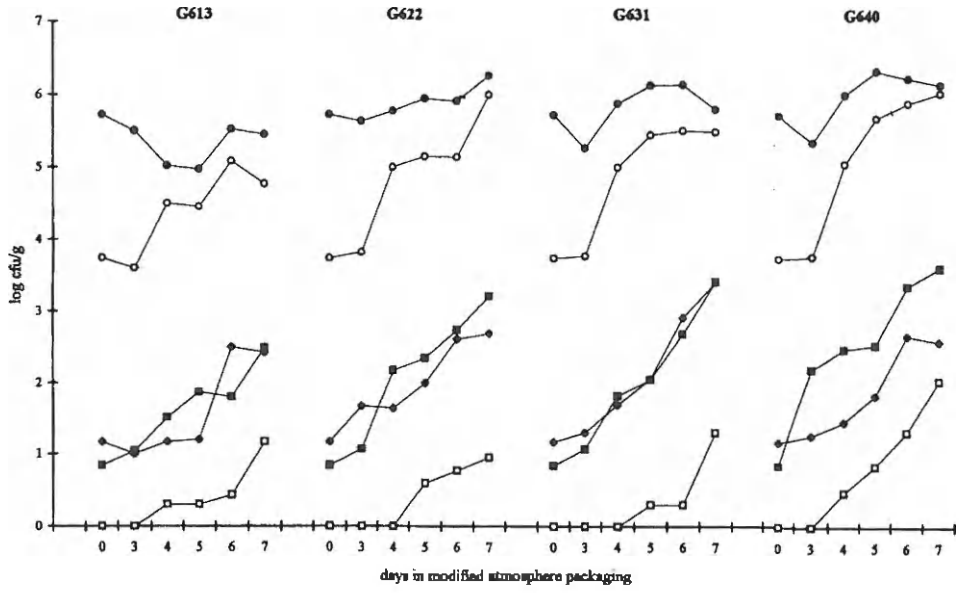

Fig. 1. Bacterial plate counts (aerobic —●—, anaerobic —○—, lactic acid bacteria —◆—, H_2S-producing bacteria —■—, *Enterobacteriaceae* —□—) on cod fillets packed under modified atmosphere (G613, G622, G631, G640) for 7 days.

aerobic bacterial plate count at the seventh day. In the gas atmosphere G613, the increase of the anaerobic count was slower compared to the other atmospheres.

Lactic acid bacteria increased by 1–2 logarithmic units during 7 days in modified atmosphere packaging (Fig. 1). There is no difference in the lactic acid bacteria plate counts among the gas mixtures.

Hydrogen sulfide producing bacteria increased 2–3 log units in 7 days starting from the third day, except in the G640 atmosphere where there was an early outgrowth (Fig. 1). In no case the count of H_2S-producing bacteria reached the spoilage limit of 10^7 cfu/g.

Incidence of *Enterobacteriaceae* was observed only after 4 days in the G613 and G640 atmosphere and after 5 days in G631 and G622 atmosphere (Fig. 1). Further growth was very slow. On the seventh day 10 cfu/g were present, except for the G640 atmosphere where growth was slightly faster and 100 cfu/g were counted finally (Fig. 1)

3.2. Chemical analysis

The proportion of CO_2 in the atmosphere of the packages decreased until day 4 because of diffusion into the fish muscle (results not shown). From day 5 on, the content of CO_2 increased again due to bacterial and enzymatic activity. Reciprocally to the CO_2 content the proportion of O_2 increased up to day 4. Due to respiration of bacteria the proportion of O_2 decreased later on.

The production of TVB was continuous in all atmospheres (Fig. 2). TMA, like TVB, is continuously produced during 7 days storage in MAP. From day 5 on, the rate of TMA production differs depending on the packaging atmosphere and it can be seen that higher levels of oxygen delay the production of TMA.

As a consequence of TVB production a slight increase in pH was noticed during the first 4 days of storage (Table 1). Diffusion of the CO_2 in the fish muscle shows a certain counter effect on the pH increase by TVB production, resulting in a stabilization of the pH around 6.7.

4. Discussion

Despite the fact that the total number of bacteria, determined in PCA at 25°C, was relatively high at day 0 ($\sim 10^6$ cfu/g) it did not increase significantly and remained below 10^7 cfu/g (Fig. 1). The carbon dioxide atmosphere, with variable proportions in oxygen, can be considered effectively inhibitory on the total aerobic flora. This composition of atmosphere is in accordance to most recommendations and practices for 'seafood' suggesting an initial concentration of CO_2 between 30 and 60% (Davis, 1993). Tiffney and Mills (1982) found that O_2 actually increased the shelf life of white fish in controlled atmosphere. Robertson (1993) proposes a mixture of 30% O_2-40% CO_2-30% N_2 for non fatty fish and 40% CO_2-60% N_2 for fatty fish. Sacks and Gore (1987) included 40%

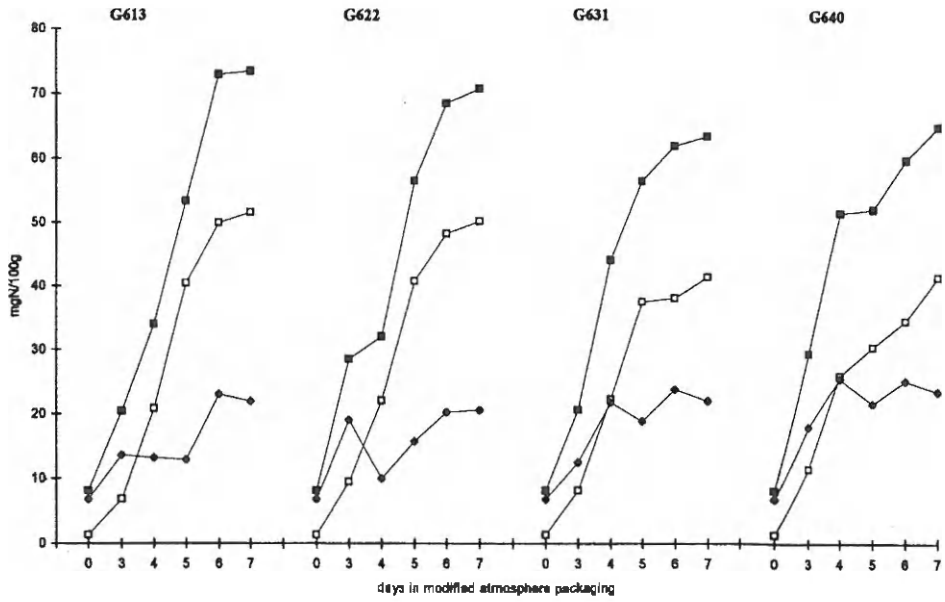

Fig. 2. TVB (—■—), TMA (—□—) and FBVN (—◆—) production in cod fillets packed under modified atmospheres (G613, G622, G631, G640) for 7 days. (FBVN = TVB-TMA).

O_2 and 60% CO_2 as an alternative for white fish. On the contrary, Kimber (1984) declared that fish requires an inert mixture of CO_2 and N_2, and that care is taken to remove any O_2.

However, significant production of TVB and TMA, which contributes to a negative effect on the organoleptic quality of the fish fillet, was noticed. Increase in TVB is mainly ascribed to TMA production rather, than to the production of other volatile bases (Fig. 2). There are not many references on the relation of the gas mixture to the spoilage by TMA production. According to Easter (1982) oxygen exerts an inhibitory effect on the TMAO-reductase activity of *Alteromonas* spp., while CO_2 has an indirect inhibitory effect on the same enzyme by reducing the pH to ~ 6. Easter et al. (1982) proved that TMAO reductase has an optimum activity

Table 1
Changes in pH values of cod fillets stored for 7 days under modified atmosphere packaging

Days	G613	G622	G631	G640
0	6.33	6.33	6.33	6.33
3	6.48	6.53	6.40	6.41
4	6.74	6.60	6.70	6.91
5	6.76	6.66	6.79	6.69
6	6.70	6.63	6.68	6.71
7	6.64	6.69	6.75	6.69

at pH 6.8. As it is demonstrated in Table 1, the pH of cod increased from the initial pH of 6.33 to ~6.7, enhancing the activity of TMAO-reductases. Therefore Vilemure et al. (1986) suggested that the storage time of cod fillets, stored in 25% CO_2-75% N_2 controlled atmosphere at 0°C, should be restricted to 7 days on the basis of TVB production. Davis (1990) used a gas mixture of 40% CO_2-30% O_2-30% N_2 for cod fillets stored at 0, 5 and 10°C and observed the TMA score increasing rapidly after 11, 4 and 3 days respectively.

In Fig. 1 it can be seen that the aerobic flora shows a facultative anaerobic behaviour and is resistant to CO_2. Lactic acid bacteria are carbon dioxide tolerant too, but referring to Hanna (1992) their growth in fish is limited for the first 14 days. The inhibition of *Enterobacteriaceae* by carbon dioxide has been reported several times in the past by other researchers (Gill and Tan, 1979; Coyne, 1933; Haines, 1993). A major part of the H_2S-producing bacteria is facultative anaerobic and has the ability of reducing TMAO to TMA (Huss, 1988). Hydrogen sulfide producing bacteria represent only a small part of the total flora in cod fillets packed in CO_2-atmosphere (Fig. 1).

By comparing the four types of gas atmosphere G640 atmosphere, (60% CO_2, 40% O_2) can be considered as the most effective for the inhibition of TMA production. The higher oxygen availability in this atmosphere leads to a low utilization of TMAO as a secondary electron acceptor. However, it has to be noted that the application of a G640 atmosphere can not entirely prevent the spoilage by TMA. Application of higher oxygen levels could possibly reduce the production of TMA but this will be on the expense of the carbon dioxide level and the antimicrobial activity of the latter.

A total aerobic plate count at 25°C of a 10^6 cfu/g, combined with the presence of only a 10^3 cfu/g of H_2S-producing bacteria, which are normally considered as TMAO-reducing organisms in fish, cannot explain the strong increase in TMA. Indeed, a high cell concentration of more than 10^8 cfu/g of *Shewanella putrefaciens* is required for production of a TMA level normally found in spoiled fish (Dalgaard, 1995). This suggests that there could be another type of bacterium in fish, not involved in the spoilage of unpacked fish, which are resistant to 60% of CO_2, are not H_2S-producing, and show a high TMAO-reducing capacity per cell unit. This conclusion is in agreement with the findings of Dalgaard et al. (1993) and Dalgaard (1995) suggesting that large cells of *Photobacterium phosphoreum* could be responsible for the TMA production and hence for the spoilage of modified atmosphere packed fish. On average, cells of *P. phosphoreum* produce several times more TMA than cells of *S. putrefaciens* (Dalgaard, 1995)

It can be concluded that packaging of cod fillets in modified atmosphere, containing 60% CO_2 and 40% O_2 (1 part of gas and 1 part of fish), and storing at 6°C have an inhibiting effect on the growth of the normal TMA- and H_2S-producing flora (*Shewanella putrefaciens*). However, these conditions are less effective on the inhibition of the growth and activity of the TMAO-reducing large cells of *Photobacterium phosphoreum*, which are not H_2S-producing, and show a high resistance against 60% CO_2. In order to decrease the TMAO-reduction by *P. phosphoreum* O_2 can be added in the packaging atmosphere.

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