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Combined Effect of Modified Atmosphere Package and Short-Wave Ultraviolet Does Not Affect *Proteus mirabilis* Growth on Rainbow Trout Fillets (*Oncorhynchus mykiss*)

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Abstract The current study investigated the effectiveness of short-wave ultraviolet (UV-C) radiation on rainbow trout fillets inoculated with *Proteus mirabilis* when combined with Modified Atmosphere Packaging technology (MAP). Rainbow trout were inoculated, packaged under different ratios of CO_2 and N_2 gases and subjected to UV-C radiation. Our study model demonstrated that at least 0.1001 J/cm^2 is necessary to significantly reduce *Proteus mirabilis* loads (reduction of 1.8 log CFU.g^{-1}) in trout fillet packaged without CO_2 gas barrier. The rainbow trout fillet packaged with CO_2 gas barrier had significantly reduced *Proteus mirabilis* load but not when associated with UV-C radiation exposure. The combined effect of UV-C and MAP at different radiation doses and ratios of CO_2 and N_2 gas did not contribute to *Proteus mirabilis* growth reduction. Overall, the use of MAP significantly reduces the penetration and effect of UV-C radiation when compared to the unpackaged control. The combination of these two technologies of food preservation does not seem to be a suitable model to extend the shelf life of packaged fish fillet.

Keywords: *freshwater fish, UV-C radiation, modified atmosphere package, Proteus mirabilis, shelf life*

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1. Introduction

Rainbow trout is one of the major fish species cultivated and marketed by commercial aquaculture, mainly due to its advantageous husbandry characteristics, nutritional properties, and wide acceptance as meat matrix by the market [1,2,3]. However, the quality of fresh trout is still a major concern for the food industry and consumers due to high perishability. Mainly due to chemical composition and microorganism activity, spoilage of fish occurs rapidly resulting in a loss of quality and shorter commercial shelf life [4,5,6,7].

Several preservation methods have been studied to control the rapid deterioration of fish meat. Ultraviolet radiation is a non-thermal technology used in food processing to inactivate pathogenic and spoilage microorganisms present on food surfaces [8,9]. The UV-C radiation (wavelength 253.7 nm) is used to obtain a germicidal effect and has been approved by the Food and Drug Administration (FDA) for use on food product surfaces [10,11,12]. This technology has some technical advantages: it is an effective and relatively inexpensive process that is easy to implement, and it does not generate chemical and radioactive residues [13].

Besides UV-C radiation, conservation methods that act over an extended period, such as Modified Atmosphere Packaging technology (MAP), have been studied for their ability to extend the commercial shelf life of products by preserving freshness and quality [14,15]. The combination of UV-C radiation and MAP may be a strategy to effectively reduce the microbial load and therefore enhance the shelf life of products. However, the packaging composition also may be significant when these two techniques are combined once certain packaging may impair the penetration of UV-C radiation and thereby reduce the effectiveness of this preservation method.

Packaging is the final step of food production and further safety treatments combined with UV-C radiation could be essential to eliminate possible contamination by instruments or personnel and to decrease the risk of possible microorganism activity [13]. Hence, studies regarding each specific microorganism and food matrix are required to evaluate the efficiency of UV-C radiation since their sensitivity may vary [16].

Although many studies have evaluated the application of UV-C in pathogenic microorganisms [9,17,18,19,20], there is a lack of information about the effectiveness of this method in *Proteus* sp. inoculated in fish meat. This microorganism may be part of the natural microbiota of fish, or its presence may be caused by contamination during food processing [21]. Moreover, these microorganisms can produce decarboxylase enzymes and are great producers of biogenic amines in fish, thereby constituting a hazard to public health [22]. Hence, we used *Proteus* sp. as a model of bacteria-producing biogenic amines in the present study.

Because there is limited scientific evidence about the efficiency of UV-C on products that are already packaged with CO_2 gas barrier packages, this study aims to evaluate the UV-C radiation effectiveness in rainbow trout fillets inoculated with *Proteus mirabilis*, packaged under gas barrier packaging with different proportions of gases (CO_2 and N_2). Furthermore, analyses of packaging materials that allows the best response when applying UV-C radiation was also addressed.

2. Material and Methods

2.1. UV-C Equipment

A stainless-steel barrel-shaped chamber was constructed to perform the experiments. Twelve UV-C lamps, six of 30W and six of 55W (OSRAM™ HNS, OFR, Munich, Germany), were distributed in interspersed positions inside the chamber. Nylon netting was used to position the samples in the geometrical center of the chamber. The intensities applied were 0.713 mW/cm^2 (30W lamps), 1.065 mW/cm^2 (55W lamps) and 1.668 mW/cm^2 (30 and 55W lamps), determined by UV radiometer (MRUR-203™, Instrutherm, São Paulo, Brazil). In order to determine the higher irradiance inside the chamber, different locations throughout the nylon net were tested by UV radiometer.

2.2. Packaging Material

In order to evaluate packaging that allows superior penetration of UV-C radiation, three types of low-density polyethylene commercial packaging were tested while empty with three doses (0.0428±0.00, 0.0639±0.00 and 0.1001±0.01 J/cm^2): (1) Laminate packaging– LP (20 cm x 30cm); (2) Packaging with CO_2 gas barrier– GB (20cm x 35cm); (3) Packaging without CO_2 gas barrier– WGB (19cm x 30cm). A radiometer was located inside the empty package and the irradiation was measured for 60 s. The measurements were performed in triplicate. Furthermore, these intensities were measured without the presence of packaging, constituting control values.

2.3. Preparation of Inoculums

One serotype of *Proteus mirabilis* (INCQS 00265) was used in this study. This serotype was obtained from the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). The inoculations were performed according to [23]. In brief, the culture was grown in Falcon tubes containing 30 mL of BHI at 37°C for 24h, and the process was repeated twice. This initial step was used to obtain bacterial cultures in the stationary phase of growth at a concentration of 8 log CFU/mL. After the final incubation, cultures were centrifuged at 1000 *g* for 15 min at 4°C. Cell pellets were washed three times with sterile phosphate buffered saline (PBS, pH 6.0). The bacterial concentration was determined by measuring the optical density at 600nm (OD600) by UV spectrophotometer (Smartspec Plus, BioRad, Hercules, CA, USA).

2.4. Fish Meat Samples and Inoculation

Two kilograms of eviscerated rainbow trout were obtained from the Trutas da Serrinha Company located in Itatiaia, Rio de Janeiro, Brazil. The samples were packed in ice (0±1°C) and transported in a Styrofoam box to the laboratory. In order to obtain fillet samples of 8 x 8 cm, the trout was filleted under sterile conditions.

Sixteen samples were aseptically packaged in vacuum package and conditioned at 4°C until inoculation, which did not exceed two hours. Each package was opened, and one milliliter of inoculum solution with OD600 (corresponding to approximately 7.0 x 10^8 cells) was inoculated onto the surface of each trout fillet. Afterwards, samples were packaged using different proportions of gases and submitted to UV radiation.

2.5. Different Gas Ratio and UV Radiation

Sixteen treatments were performed using either separate or combined conservation methods. Nine treatments were performed by combining three doses of UV radiation (0.0428±0.00, 0.0639±0.00 and 0.1001±0.01 J/cm^2) with three different proportions of gases (30%, 50% and 70% of carbon dioxide; the remaining volume was completed by nitrogen gas and vacuum was performed prior to the addition of modified atmosphere gases (TECMAQ, Vacuum sealer, AP 450). Three treatments involved UV radiation alone (at three treatment levels with doses of 0.0428±0.00, 0.0639±0.00 and 0.1001±0.01 J/cm^2) and three involved gas ratios alone (at 30%, 50% and 70% of carbon dioxide). The control sample (positive control) was inoculated and packaged without UV radiation or modified atmosphere. UV exposure was administered for 60 s in all treatments. Each sample was placed in a central area (10 x 40 cm^2) of the unit and irradiated simultaneously on both surfaces. After packaging at different gas proportions and after administering UV radiation doses, bacteriological tests were performed.

2.6. Bacteriological Analysis

A swab method was performed on the surface of fillets with the aid of a sterilized mold by autoclaving (2 x 5 cm). The swabs were placed in tubes containing saline and

6-fold serial dilutions. An amount of 1 mL was removed from each dilution and cultured by the pour plate technique in triplicate on Salmonella-Shigella agar. The plates were incubated at 37°C for 48 h. One additional fillet was tested for the initial presence of *Proteus* species (negative control). Culture forming units were counted and expressed as log $CFU.g^{-1}$ [24].

2.7. Statistical Analysis

Two-way analysis of variance (ANOVA) with repeated measures on two factors (4 x 3; packaging material x UV-C doses) was used to identify differences in absorption of UV-C doses radiation for different types of packaging. A two-way ANOVA with repeated measures on two factors (4 x 4; UV-C doses x proportion of gases) was utilized to identify differences in the reduction of bacterial load of *Proteus mirabilis* when submitted to doses of UV-C radiation with or without the use of different proportions of gases. When a significant *F* was found, additional post-hoc tests with Bonferroni adjustment were performed. Statistical significance was set at the 0.05 level of confidence. All analyses were performed using the commercially available statistical package XLSTAT version 2013.2.03 (Addinsoft, Paris, France).

3. Results

All packages allowed the penetration of UV-C radiation with the exception of the laminate packaging that did not allow the passage of UV-C radiation regardless of intensity. The presence of packages with and without CO_2 gas barrier significantly reduces the penetration of UV-C which demonstrates that packaging materials significantly decrease the passage of UV–C radiation. Packages with and without CO_2 gas barrier only differed significantly when the highest UV-C dose (0.1001±0.01 J/cm^2) was applied (Table 1).

Table 1. Residual UV-C doses (J/cm^2) for different packaging (WGB, GB and LP)

UV-C Doses (J/cm^2)	Packages			
	UP	WGB	GB	LP
0.0428	$0.0428±0.00^{aA}$	$0.0306±0.01^{bA}$	$0.0316±0.00^{bA}$	0.000±0.00
0.0639	$0.0639±0.00^{aB}$	$0.0454±0.00^{bB}$	$0.0435±0.01^{bB}$	0.000±0.00
0.1001	$0.1001±0.01^{aC}$	$0.0781±0.00^{bC}$	$0.0692±0.01^{cC}$	0.000±0.00

Values are means ±SD. UP: unpackaged; WGB: without CO_2 gas barrier packaging; GB: CO_2 gas barrier packaging; LP: laminate packaging. Different lowercase letters indicate significant differences (P <0.05) for values within a row. Means with different capital letters show significant differences (P <0.05) between values within a column.

The log reduction of *Proteus mirabilis* on rainbow trout fillets with UV-C radiation at different doses and gas ratios are shown in Table 2.

Proteus mirabilis only significantly reduced when the highest dose of UV-C (0.1001±0.01 J/cm^2) was applied at MAP with 100% of N_2 (reduction of 1.8 log $CFU.g^{-1}$), and when the gas barrier of 70% CO_2 was applied in the package with no UV-C treatment. The combined effect of UV-C and MAP at different radiation doses and ratios of CO_2 and N_2 gas did not interfere at *Proteus mirabilis* growth.

Table 2. Log reduction of *Proteus mirabilis* (UFC/mL) on rainbow trout fillets with different UV-C light doses and gas ratios

Gas Ratio* (%CO_2)	UV-C doses (J/cm^2)			
	0	0.0428	0.0639	0.1001
0	$0.16±0.40^{aA}$	$0.65±0.47^{abA}$	$0.90±0.70^{abA}$	$1.80±0.11^{bA}$
30	$0.86±0.76^{aAB}$	$1.10±0.72^{aA}$	$1.60±0.70^{aA}$	$1.37±0.58^{aA}$
50	$1.30±1.22^{aAB}$	$1.09±0.53^{aA}$	$1.13±0.47^{aA}$	$1.31±0.67^{aA}$
70	$1.28±0.25^{aB}$	$1.18±0.38^{aA}$	$1.28±0.42^{aA}$	$1.58±0.72^{aA}$

Values are means ±SD. Different lowercase letters indicate significant differences (P <0.05) for values within a row. Different capital letters indicate significant differences (P <0.05) between values within a column. * the remaining volume was completed by nitrogen gas (N_2).

4. Discussion

As demonstrated by [25] an extended shelf life of freshwater fish by using combinations of 40-60% CO_2 to inhibit deteriorating compounds and bacterial growth. However, others studies demonstrate that lower (30%) and higher (70%) concentrations of this gas also can have the positive effect of the inhibiting bacterial growth [26,27,28]. Due to the results of the mentioned studies, we decided to test low (30%), medium (50%) and high (70%) concentration of CO_2 gas in our experiments.

On the other hand, the UV-C equipment available did not support the inclusion of powerful lamps. Hence, we had to limit the UV-C doses to the maximum of 0.1001±0.01 J/cm^2. The exposure time of 60 s was set based on previous results of [29], who demonstrated that *E. coli* and *Listeria monocytogenes* were reduced by 1 log when applying UV-C radiation for this period. The germicidal effect of UV-C has been attributed to cross-linking between the nucleic acid thymine and cytosine of DNA in microorganisms caused by absorption of UV-C radiation. This bond results in the blocking of DNA transcription and replication, inhibiting cell growth, leading to cell death and decreasing the bacterial load on the food surface [30,31].

Laminated packages have been previously used as a strategy of food conservation to reduce the production of free radicals derived from oxidation of unsaturated fatty acids by exposure to solar radiation, among other factors [32]. However, our results demonstrated that laminated packaging blocks the passage of ultraviolet rays by 100%, even at the highest UV-C dose (0.1001±0.01 J/cm^2) (Table 1). This phenomenon may be due to reflectance from the lamination of the packaging, where the incident radiation is equal to reflected radiation, hindering or even preventing the passage of UV-C rays [33,34,35]. In this context, laminated packaging should not be used for products to be subjected to the process of UV-C radiation.

Overall, MAP significantly reduces the penetration power of the UV-C, and this should be considered when choosing the UV-C dose to reach the residual effect over the food inside the package. Interestingly, our results showed that packaging without a CO_2 gas barrier (WGB) allowed a significantly higher passage of UV-C in comparison with packaging with CO_2 barrier (GB) only when it was exposed to the highest radiation dose of

0.1001±0.01 J/cm^2 (Table 1). It might be possible that the radiation dose of 0.0428 and 0.0639 (J/cm^2) were not sufficiently powerful to receive some inhibitory influence of the CO_2 gas barrier and cause significant reduction of the final residual concentration.

Radiation of 0.1001±0.01 J/cm^2 also affected bacterial growth on inoculated trout fillets at packaging without CO_2 gas barrier, resulting in a reduction of 1.8 logs (CFU.g^{-1}) of *Proteus mirabilis* (Table 2). Different doses of UV-C radiation, when applied in combination with CO_2 and N_2 gas packaging barrier, did not potentialize the reductive effect of the bacterial growth in all the three doses. These results might suggest that the CO_2 gas presence in the MAP have some inhibitory influence over the toxically effect of the UV-C radiation over bacteria. Curiously, *Proteus mirabilis* loads significantly reduced with the increase to 70% of CO_2 proportion only in gas barrier packaging not exposed to UV-C radiation (Table 2). That result also demonstrated that the combined effect of MAP with UV-C radiation might not be satisfactory to increase the trout fillet shelf life by reducing *Proteus mirabilis* loads.

Fish preservation conditions as freezing, thawing and the presence of bacteria are the major factors in the formation of biogenic amines. Biogenic amines in fish and fish products are one of the best indicators to determine the poor-quality raw material. The presence of high levels of biogenic amines in foods may cause migraine, headache, gastric and intestinal problems and pseudoallergic responses. In this context, *Proteus mirabilis* is one of the main enterobacteria that can decarboxylate the amino acids to biogenic amines [36]. Reducing loads of this bacteria in food products increases the final quality of the raw products and decreases the risk of intoxication by biogenic amines producing.

There are no studies that examine the effectiveness of UV-C radiation combined with MAP with different gas proportions against *Proteus mirabilis* and fish meat. However, our results are in agreement with previous studies that showed a reduction of other bacterial groups and food matrices. UV-C exposure of up to 0.192 J/cm^2 for 32 seconds reduced *E.coli*, *S.* Enteritidis and other Enterobacteriaceae on chicken skin by 0.77, 1.01 and 0.30 log CFU/g, respectively, and on skinless chicken fillet by 0.98, 1.34 and 1.29 log CFU/g for the same microorganisms, respectively. As reported by [8] an initial population reduction of *S.* Typhimurium by 1.19 log CFU/g in chicken breast submitted to 0.05 J/cm^2. As observed by [37] a reduction of 1.403 log CFU/g in the pathogenic *E. coli* strain 0157:H7 in liquid egg white when exposed to UV-C radiation of 1.57 J/cm^2 for 20 min.

As noted, the reduction of the bacterial load varies depending on the microorganism and food matrix. Characteristics of the food matrix and the UV-C source, such as food surface topography and composition, microorganism locality and the power and wavelength of the UV-C lamp, can interfere with the efficiency of UV-C radiation to reduce superficial bacterial load [13,16,34,38]. Furthermore, food matrices with a higher load of gram-negative bacteria respond better to UV-C radiation since these microorganisms have only one layer or several thin layers of the outer membrane leading to lower resistance to UV-C radiation than gram-positive bacteria [37,39].

5. Conclusion

The reason why the combination of UV-C and MAP does not improve the reduction of microbial growth in packaged trout fillet remains inconclusive. Indeed, the combination of these two technologies of food preservation does not seem to be a suitable model to extend the shelf life of packaged fish fillet. In future, novel and more detailed experiments involving different microorganisms, MAP and UV-C will be performed to understand this phenomenon better.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper

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