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Colour Analysis Method Applied to Browning of "*Pesto*" Spreads Preserved With Organic Acids And Lactoferrin

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Abstract: Natural metal-chelating protein, lactoferrin, potentially influences lipid oxidation, microbiology and browning (attributed to combined enzymatic and non-enzymatic oxidation) of minimally-processed green vegetable-based food emulsions, depending on milieu conditions. We have compared the effect of combinations of antioxidative and antimicrobial agents on microbial count, lipid oxidation and browning in fresh basil-based ("*pesto*") spreads. Browning was determined by colour lightness changes, applying colour evaluation, by means of multivariate image analysis. Addition of 80 and 400 mg/kg lactoferrin reduced primary lipid oxidation products accumulation (peroxide value) in separated oil phase of the product, as well as *Lactobacillus* sp. counts, after 1 month of sample storage at 4°C. Oxidation induction time (OIT), determined by differential scanning calorimetry (DSC) and browning rate were slightly negatively affected in samples containing lactoferrin and 0.5 g/kg of ascorbic acid.

Key words: colourgram, basil, emulsions, antioxidants, browning, DSC, lactoferrin.

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Introduction

Consumer requirements for minimally processed, but safe food, having preserved organoleptic properties is a guideline to industrial processing modifications. Fortification with natural antimicrobial and antioxidative agents, as an alternative to thermal treatment for controlling food borne pathogens, potentially influences microbiology, lipid oxidation and browning (attributed to combined enzymatic and non-enzymatic oxidation) of minimally-processed green vegetable-based food emulsions. Fresh basil-based spreads, similar to non heat-processed "*pesto*", an Italian pasta sauce, whose main ingredients are basil, olive and sunflower oil, cheese and walnuts, are identified as a benchmark to test the antioxidative and antimicrobial properties of lactoferrin, a metal-chelating protein, isolated from whey, which is an antimicrobial agent and which, also, influences lipid oxidation (Chiu and Kuo 2007, Huang *et al*. 1999). "*Pesto*", obtained by the traditional recipe, is limited to the very short shelf life. For this reason, pasteurisation or sterilisation and/or lowering of pH are needed for industrial production, but these treatments lead to colour, taste and flavour degradation. Lactoferrin, the main iron-binding protein present in mammalian milk, has antimicrobial activity against a wide range of Gram-positive and Gram–negative bacteria, fungi and parasites. Furthermore, as a component of innate immunity, lactoferrin is an antiinflammatory, immunomodulating, antioxidative, anti-cancer and antiviral agent and could enhance the growth of probiotic bacteria, like *Bifidobacterium* (Farnaud and Evans 2003). Several studies indicated the potential value of using lactoferrin as a natural bactericidal agent in food industry. However, presence of divalent cations, like Ca^{2+} and Mg^{2+}, at concentration 1-5 mM, reduced its antimicrobial activity, probably by inducing changes in lactoferrin tertiary structure (Al-Nabulsi and Holley 2007). Other milieu conditions, such as pH, elevated NaCl or iron, inappropriate citrate/bicarbonate ratio, could markedly diminish the antimicrobial activity of lactoferrin by enhancement of cationic charge interactions or facilitating apo/holo lactoferrin transition (Naidu 2002, Wakabayashy *et al*. 2006). The objective of the present study was to assess the effect of combinations of antioxidants and antimicrobial agents (ascorbic, citric and lactic acid, NaCl and lactoferrin), on lipid oxidation and microbial count of fresh basil-based spreads. Oxidative browning was determined by colour lightness changes, applying colour evaluation, by means of multivariate image analysis (Antonelli *et al*. 2004). Software was developed for processing of digitalized images in the form of one-dimensional signal, describing the colour content. The coloured, red, green and blue (RGB) images were taken with a common digital CCD camera. A signal describing the colour intensity of each acquired image is created as the continuous sequence of frequency distribution curves of RGB.

Materials and methods

Preparation of basil-based spreads. Basil-based spreads similar to Italian "*pesto*" sauce, were prepared from fresh basil leaves, which were treated with hydrogen peroxide and/or short temperature shock during washing (Lin *et al*. 2002) combined with xylitol and lactoferrin as potential sanitizers. Basil, type „Basilico

Genovese", (*Ocimum basilicum* L.), was cultivated in the experimental garden at Faculty of Agriculture, Belgrade, Serbia. The product contained virgin olive oil, refined sunflower oil, cheeses, such as parmesan and kachkaval, whey, walnuts, sunflower seeds, salt and garlic, as well as organic acids (ascorbic, citric and/or lactic acids) (Sigma-Aldrich, Germany) and lactoferrin (bovine lactoferrin, 95% protein, DMV International, Veghel, The Netherlands) as regulators of acidity, antioxidative and antimicrobial agents. The product was packaged in glass jars of 150g.

Physico-chemical analysis. After a certain period of storage (0 and 30th days), the samples of "*pesto*" spread were frozen at -70^{o}C for ten days. After defreezing, the content was centrifuged at 26100 g for 30 minutes and analyzed for oxidative stability of separated oil phase (Jacobsen *et al*. 1998). The development of primary products of lipid oxidation was followed by peroxide value (PV) (ISO 3960: 1998) and free fatty acids were analyzed by acid value (AV) (ISO 660: 1996) in the separated oil phase of "*pesto*" spread. The oxidative stability of "*pesto*" oil phase, as the criterion of total oxidative stability, was analyzed according to the method of differential scanning calorimetry (DSC), at isothermal conditions (105 ^{o}C) in oxygen current, purge flow 50ml/min, (Velasco *et al*. 2004) on "Tzero" technology calorimeter, DSC Q 1000, TA Instruments, (Delaware, USA), and expressed as oxidation induction time (OIT_{DSC}) according to standard method ASTM E 1858-08. From resulting exotherm, oxidation induction time (OIT_{DSC}) was determined by TA Instruments Universal analysis 2000 software. pH was determined using a pH-metre (Consort, C 830, USA) with a viscous emulsion electrode. All experiments were done in duplicate.

Microbiological analysis. Microbial population was evaluated after fresh basil washing treatments (total bacterial count and yeast and mould count). Microbiological counts in "*pesto*" spreads were made on the 1st and 30th day of storage at 4^{o}C. Twenty grams of each sample were diluted with 180 ml of sterile solution of 2% sodium citrate. For the scoring of total bacterial count, 1 ml of diluted samples were spread on a plate with nutrient agar (Torlak, Ref 300763, Serbia), and incubated for 72 h at 30^{o}C. For the determination of *Lactobacillus* sp., diluted samples were incubated on MRS medium (Himedia M64, India) and counted after 48 h at room temperature. The number of yeasts and moulds was determined after 5 days of incubation on Sabouraud Malthose agar (Torlak 399405, Serbia) at room temperature. All experiments were done in duplicate.

Colour analysis. Colour images of basil-based "*pesto*" spreads were captured by a Canon PowerShot A550 and A590 CCD camera, which is a common digital camera for home use. All the acquired images were 24 bit *RGB* (16.8 millions of colours) with a 1024 x 768 spatial resolution. The macro function of the digital camera has been used to cover a scene area of approximately Ø10 cm. Each "*pesto*" sample was smeared to an approximately constant 3-5 mm thickness and to an area sufficient to cover completely the image scene, in order to avoid the presence of background in the image. Then, it was placed on a white paper napkin set on a flat white painted surface, 15 cm below the digital camera, which was placed on a fixed position. Paper napkins were used in order to absorb the excess of oil and therefore to avoid undesired reflection effects. With this setup, it was possible to capture

images with negligible shadows and without specular reflections. There were 9 different pesto spreads to be observed, and the images of each different spread of pesto were taken periodically by camera 22 times during the period of approximately 320 minutes.

The acquired RGB images were transferred to a personal computer in the form of .jpeg compressed image files by means of the software provided with the digital camera. The size of the imported .jpeg images ranged from 900 to 1250 KB for the 9 x 22 = 198 images of the data set. For the elaboration of the images, every image file is imported in the originally developed computer program as a RGB picture. This program transforms digital picture data to the three-dimensional array.

The first step in the elaboration of every single digital *RGB* image with size {r, c}x{ R, G, B} (where *r* is the number of pixel row, *c* is the number of pixel column and *R, G* and *B* channels) consists of unfolding it to a three-dimensional matrix of the *R, G* and *B* values (variables). Since the imported files are 24 bit colour images, each one of the three *R, G* and *B* "slices" of the 3D array are (24/3) 8 bit greyscale images, and therefore the three *R, G* and *B* variables can assume all the integer values in the range 0-255. After this first step these coordinates were normalized, i. e., divided by 255, in order to be represented as values varies from 0 – 1.

Then, a series of quantities derived directly by the normalized *R, G* and *B* variables with simple algebraic operations are calculated for every pixel: lightness (L), which is the sum of *R, G* and *B,* divided by 3 (*L*=(*R*+*G*+*B*)/3) and the three relative colours, i.e. relative red (*RR=R/(L·3)*), relative green (*RG=G/(L·3)*) and relative blue (*RB=B/(L·3)*), which are ratios between the *R, G, B* values and lightness, *L*. It should be noticed that these values are also normalized, i. e., varies from 0 to 1. The relative values have been considered since a particular colour, e.g. red colour does not correspond to a high absolute value of the corresponding *R* variable, but to a high value of the ratio between *R* and *L*.

The hue, saturation and intensity *(HSI)* values are also calculated, using the developed computer program, for the conversion from the *RGB* to the *HSI* colour space. The *I* values are computed for every pixel as the maximum value between *R, G,* and *B:*

$$I = \max(\mathrm{R}, G, B) \tag{1}$$

while the *S* values are given by:

$$S = \frac{\max(R,G,B) - \min(R,G,B)}{\max(R,G,B)} \tag{2}$$

H value is defined by a quite complex algorithm, difficult to represent in an easily readable analytical form, in a way that it varies from 0 to 1 with the corresponding colour passing from red through yellow, green, cyan, blue, and magenta, and then back to red. The Visual Basic procedure should be expressed as follows:

```
x1 = (G - R) / Sqr(2): y1 = (2 * B - R - G) / Sqr(6)
If x1 = 0 And y1 = 0 Then Arg = 0
If x1 = 0 And y1 >= 0 Then Arg = Pi / 2
If y1 = 0 And x1 < 0 Then Arg = Pi
If x1 = 0 And y1 < 0 Then Arg = -Pi / 2
If x1 > 0 Then Arg = Atn(y1 / x1)
If x1 < 0 And y1 >= 0 Then Arg = Pi - Atn(-y1 / x1)
If x1 < 0 And y1 < 0 Then Arg = -Pi + Atn(-y1 / -x1)
H = Arg * 180 / Pi + 150
If S = 0 Or I = 0 Then H = 0
If H < 0 Then H = H + 360
If H >= 360 Then H = H – 360
```

After normalization of H value, all above mentioned variables (*R*, *G*, *B*, *L*, *RR*, *RG*, *RB*, *H*, *S*, *I*) fall within the range of 0 to 1. Then, a frequency distribution vector with a length of 256 elements is calculated over all the single pixels (*r* x *c*), for each of the considered variables: *R, G, B, L, RR, RG, RB*, *H, S, I.*

The PCA analysis was evaluated in above mentioned program, using the score matrix of raw unfolded matrix, the score matrix of mean centred score unfolded matrix, and the score matrix of autoscaled unfolded RGB matrix. The element of raw matrix were: $R(i)$, $G(i)$, $B(i)$, $i = 0,\ldots, 255$. Mean centred matrix was evaluated by evaluating the subtracting the mean value of each colour vector to each element of the vector.

The autoscaled matrix was evaluated by dividing the mean centred matrix vectors with standard deviations for each vector.

The evaluation of covariance and correlation matrix for raw, mean centred and autoscaled matrix followed, with the evaluation of eigenvectors and eigenvalues of covariance matrix. The eigenvectors of covariance matrix for all three models were treated as loading vectors in PCA analysis. The used equations for calculation of covariance and correlation were:

$$\mathrm{cov}_{RG}(R,G) = \frac{\sum_{i=0}^{255}\left(R(i)-RMEAN\right)\left(G(i)-GMEAN\right)}{255}, \tag{3}$$

$$corr_{RG}(R,G) = \frac{\sum_{i=0}^{255}\left(R(i)-RMEAN\right)\left(G(i)-GMEAN\right)}{\sqrt{\sum_{i=0}^{255}\left(R(i)-RMEAN\right)^2 \cdot \left(G(i)-GMEAN\right)^2}} \tag{4}$$

The eigenvalues (λ) of evaluated covariance matrix were calculated, as usually, using the equation:

$$\det(COV - \lambda \cdot I) = 0, \tag{5}$$

where, *COV* is the covariance matrix, and *I* is identity matrix. The evaluation of roots for equation (5), was implemented, using Newton's iterative method for finding roots of function $-a\lambda^3 + b\lambda^2 + c\lambda + d = 0$:

x = ((3c/a) – (b²/a²))/3: y = ((2b³/a³)–(9bc/a²) + (27d/a))/27
z = y²/4 + x³/27
i = sqrt(y²/4 - z): j = -i^(1/3): k = arccos(-(y/2i))
m = cos(k/3): n = sqrt(3)*sin(k/3): p = -(b/3a)
Eig1 = -2j*m + p: Eig2 = j *(m + n) + p: Eig3 = j*(m - n) + p

The eigenvectors of covariance matrix are then calculated as:

$$P = COV - \lambda \cdot I\,, \tag{6}$$

and it should be calculated for all three models, raw, mean centred and autoscaled.

The score matrix is evaluated as:

$$T = X \cdot P\,, \tag{7}$$

where *X* is the raw, mean centred and autoscaled data matrix, *T* is the score model and *P* is the loading vectors (eigenvalues) of raw, mean centred and autoscaled model.

There are 3 score vectors for 3 colour coordinate, and there are 3 different PCA models (raw, mean centred and autoscaled). These vectors are joined in 256 x 9 matrix, and added to above formed 256 x 10 matrix.

The final form of colourgram is done after normalization of loading vectors and eigenvalues of three PCA models.

The resulting vectors are joined to PCA calculated matrix, which forms a one-dimensional signal, called *colourgram,* with the length of (256 x 19 + 36) = 4900 numbers, which describes the colour properties of the image.

In this work, only lightness (*L*) was used to represent data recorded during the experiment, and these data are represented in relative form, i.e., lightness was divided by initial lightness:

$$L_{rel} = \frac{L}{L_{init}} \cdot \tag{8}$$

Results and Discussion

The efficacy of combination of hydrogen peroxide and temperature in killing microorganisms (Lin *et al*. 2002) as well as temperature shock combined with other natural microbicidal agents, lactoferrin (Chiu and Kuo 2007, Huang *et al*. 1999, Farnaud and Evans 2003, Al-Nabulsi and Holley 2007, Naidu 2002, Wakabayashy *et al*. 2006) and xylitol (Tapianen *et al*. 2001) was tested on fresh basil leaves used for preparation of basil-based spreads, similar to "*pesto*" sauce. The process of basil leaves washing, combining the treatment with 2% H_2O_2 and temperature shock at 50°C for 1.5 minutes, significantly lowered the number of microorganisms, both total bacterial count and yeast and mould count, on fresh

basil, Figure 1, (2a, 2b), as it was previously observed on green lettuce leaves (Lin *et al*. 2002). Hydrogen peroxide combined with mild heat, followed by intensive rinsing with water, was proven to be effective in reducing both spores and vegetative bacteria and fungi. Lactoferrin, at the level of 10g/kg, only slightly decreased the total bacterial number, but did not affect yeast and mould count, Figure 1c. A combination of two temperature shocks, at 50°C and 90°C, in the absence of H_2O_2, led to the complete decrease in the number of viable microorganisms on basil leaves, both total bacterial count and yeast and mould count, Figure 1. (3a, 3b).

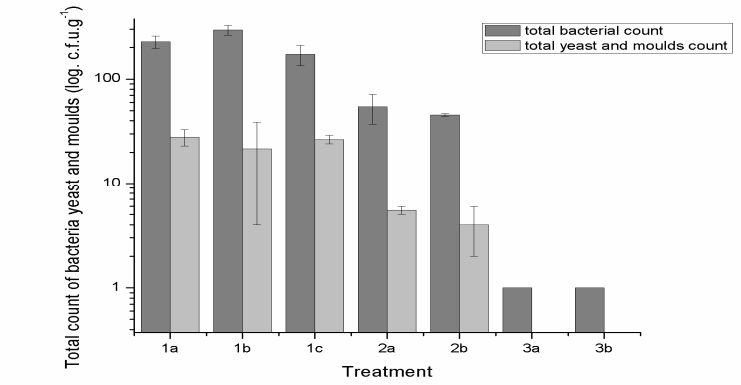

Fig.1. Total count of bacteria, yeast and moulds (c.f.u.g^{-1}) on fresh basil leaves after washing (dark grey tabs - total bacterial count, light grey tabs - total yeast and mould count). Washing in: **1a.** tap water at 20°C; **1b**. tap-water at 20°C, 2% xylitol; **1c.** tap-water at 20°C, 10 g/kg lactoferrin; **2a**. 2% H_2O_2 in deionized water at 50°C; **2b.** 2% H_2O_2 in deionized water at 50°C, 2% xylitol; **3a.** deionized water at 50°C, steam at 90°C, **3b.** deionized water at 50°C, steam at 90°C, 2% xylitol. Error bars represent SD.

The microbiological and physico-chemical stability of the fresh basil-spreads, similar to "*pesto*" sauce, treated with several combinations of antioxidative and antimicrobial substances, was evaluated. Treatments 1- 9 were designed as reported in Table 1. Nine combinations were obtained by modulating two levels of ascorbic acid, one level of citric acid, lactic acid and NaCl and three levels of lactoferrin. The concentration of organic acids were chosen to obtain three pH levels: 5.2-5.3, 4.9 and 4.4, since low pH is not favourable to the growth of many pathogens (Fabiano *et al*. 2000), including spores of *Clostridium botulinum*, type A and B. Lactobacilli and yeasts are, on the contrary, acid tolerable. By-products of the lactobacilli metabolism could be beneficial to microbiological safety, but their overgrowth could lead to the fermentation of the product. The main contribution to the bacterial load in the final product was from the fermented cheese. Lowering of the pH at 4.9, by addition of 1g/kg ascorbic and 1.5 g/kg citric acid (treatments 6 and 7) or 4.4, by addition of 1g/kg ascorbic acid,1.5 g/kg citric acid and 3.6% lactic acid (treatments 8 and 9), decreases total bacterial count, after 30 day of storage at 4°C, Figure 2.

Tab. 1. Treatments of basil-based "pesto" spreads**.** Nine combinations were obtained by modulating levels of ascorbic acid, citric acid, lactic acid, NaCl and lactoferrin

No.	Treatment	pH
1.	0.5g/kg ascorbic acid, 0.4% NaCl	5.2
2.	0.5 g/kg ascorbic acid, 0.4% NaCl, 80 mg/kg lactoferrin,	5.3
3.	0.5 g/kg ascorbic acid, 0.4% NaCl, 400 mg/kg lactoferrin	5.3
4.	0.5 g/kg ascorbic acid, 0.4% NaCl, 2000 mg/kg lactoferrin	5.2
5.	0.5 g/kg ascorbic acid, 3% NaCl	5.3
6.	1 g/kg ascorbic acid, 1.5 g/kg citric acid, 0.4% NaCl	4.9
7	1 g/kg ascorbic acid, 1.5 g/kg citric acid, 0,4% NaCl, 400 mg/kg lactoferrin	4.9
8.	1 g/kg ascorbic acid, 1.5 g/kg citric acid, 0.4% NaCl, 3.6% lactic acid	4.4
9.	1 g/kg ascorbic acid, 1.5 g/kg citric acid, 0.4% NaCl, 3.6% lactic acid 400 mg/kg lactoferrin	4.4

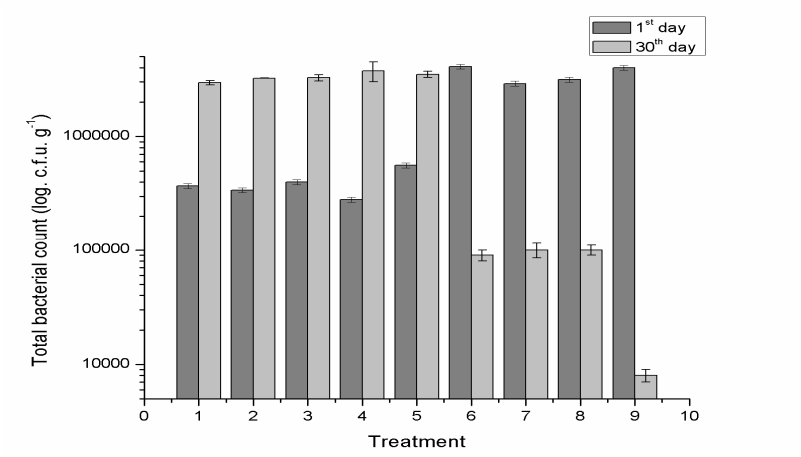

Fig. 2. Total bacterial count (log c.f.u.g^{-1}) in basil-based spreads ("*pesto"*) on the 1st and after 30 day of storage at 4°C. Dark grey tabs – 1st day, light grey tabs – 30th day. Treatments 1 – 9 were as presented in Table 1. Error bars represent SD.

Lactoferrin, at concentrations of 80 or 400 mg/kg at pH 5.2-5.3 (treatments 2 and 3) decreased lactic acid bacteria count in basil-based spreads, Figure 3. The effect of the similar levels of lactoferrin on total bacteria plate count and lactic acid bacteria count was observed in ground pork during storage of 9 days (Chiu and Kuo 2007).

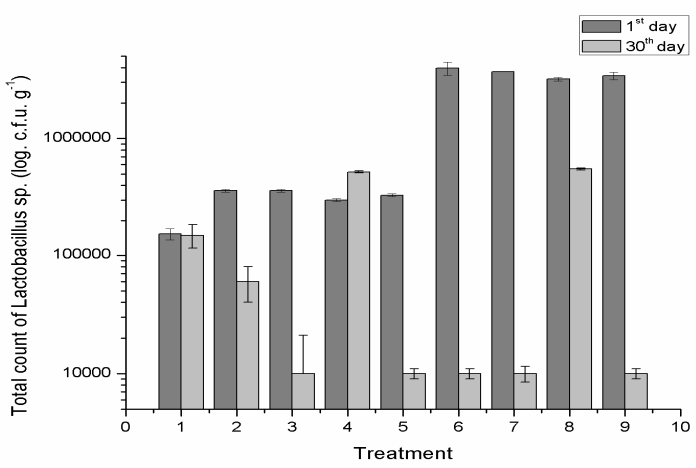

Fig. 3. Total count of *Lactobacillus* sp. (log c.f.u.g^{-1}) in basil-based spreads ("*pesto*") on the 1st and after 30 day of storage at 4^{o}C. Dark grey tabs – 1st day, light grey tabs – 30th day. Treatments 1 – 9 were as presented in Table 1. Error bars represent SD.

Initial yeast and mould number were higher at lower pH, as expected because of their acid tolerance. During the first 15 days of storage at 4^{o}C their number increased (data not shown), and then decreased at day 30, Figure 4.

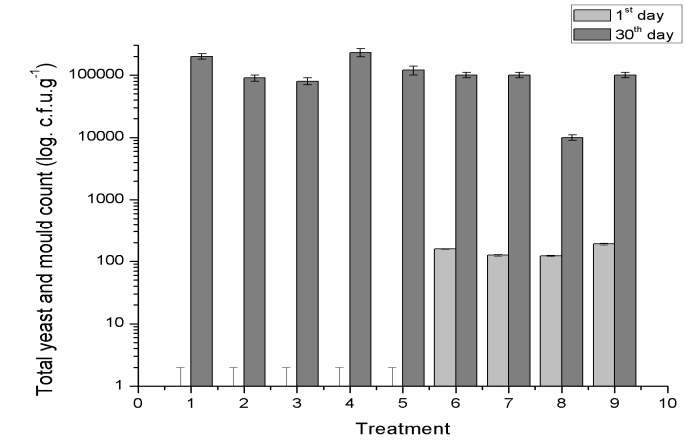

Fig. 4. Total yeast and mould count (log c.f.u.g^{-1}) in basil-based spreads ("*pesto*") on the 1st and after 30 day of storage at 4^{o}C. Light grey tabs – 1st day, Dark grey tabs – 30th day. Treatments 1 – 9 were as presented in Table 1. Error bars represent SD.

Oxidative stability of separated oil phase of basil-based spreads was also monitored We analyzed the primary oxidation products of lipids (peroxide value, PV), acid value (AV), pH, Table 2, as well as the total oxidations of lipids in the separated oil phase of the product, by the method of differential scanning calorimetry, expressed as oxidation induction time (OIT_{DSC}) for 30 days at 4^{o}C.

The process of basil wash, which combines the treatment by hydrogen peroxide and temperature shock, significantly raises (from 0-0.2 to 0.8-2.2) the PV of the final product containing 0.5g/kg of ascorbic acid. This effect was followed by the decrease of total oxidation time by differential scanning calorimetry (OIT_{DSC}), as well as decreased intensity of product browning (data not shown). In treatments 1 – 5, pH 5.2 - 5.3, ascorbic acid (0.5g/kg) and lactoferrin at concentrations of 40-2000mg/kg were added as antioxidants. The effect of natural metal chelator, lactoferrin on PV, was not observed on day 0, yet on day 30, at concentrations of 80 and 400 mg/kg (treatments 2 and 3), it came to the decrease in peroxide value, related to the control. It is known that lactoferrin can act prooxidatively[2] at high concentrations, which can explain the absence of decrease in peroxide value, at 2000 mg/kg of lactoferrin, Table 2. It was noticed previously that the increase in ascorbic acid concentration decreases the PV in mayonnaise during storage (Pavlović *et al*. 2008,). This effect was, also, obtained in treatments 6 and 7 in „*pesto*" spreads (1g/kg of ascorbic and 1.5 g/kg of citric acid), after 30 days of storage. The lowering of pH, by adding lactic acid, (treatments 8 and 9), enhance, however, peroxide values after 30 days, compared to the treatments 6 and 7, which could be related to the effect observed in mayonnaise (Jacobsen and Timm 2001), that lower pH (3.8 - 4.2) influenced the increase in lipid oxidation. This effect is attributed to the reduction of Fe^{3+}, and the breaking of Fe bridges on the interface of oil droplet. The increase of acid value (and corresponding decrease in pH) was generally observed between the 0th and 30th day and could be mostly attributed to the influence of lactobacteria, originating from cheese.

The OIT_{DSC} analysis from two separate experiments, after 30 days, did not show correlation with peroxide value. On day 0, a small decreasing trend of OIT_{DSC} with addition of lactoferrine was observed in treatments 1-4, Table 2. The OIT_{DSC} of pesto spread, generally, increased during storage of 30 days, which was contrary to the effects observed in whey salad dressings, in which OIT_{DSC} was found to decrease with the time of storage (Lin *et al*. 2002), corresponding the lipid oxidation. The effect could be attributed to water-soluble antioxidative compounds of basil origin that, probably, transfer to the oil phase in this period.

Tab. 2. Peroxide value, (PV) (mmol O_2/kg), acid value, (AV) and oxidation induction time (OIT_{DSC} at 105°C) (min.), of basil-based spreads during 30 day of storage at 4°C, (determined in separated oil phase) - the effect of 9 treatments (combinations of ascorbic, citric, lactic acid, NaCl and lactoferrin), as described in Table 1. Values represent means ± standard deviation, (SD)

Treatment no.	PV 0^{th} day	PV 30^{th} day	AV 0^{th} day	AV 30^{th} day	OIT_{DSC} 0^{th} day	OIT_{DSC} 30^{th} day
1.	1.10±0.02	1.61±0.05	1.03±0.04	1.37±0.02	177.8±4.5	248.2±7.0
2.	0.93±0.06	0.89±0.01	0.95±0.03	1.38±0.05	118.7±4.4	226.2±5.7
3.	0.98±0.04	0.34±0.03	0.98±0.02	1.42±0.03	134.5±4.3	225.9±8.1
4.	0.99±0.17	1.21±0.02	1.01±0.05	1.34±0.06	143.2±3.2	220.5±4.8
5.	1.08±0.01	0.47±0.05	1.04±0.05	1.47±0.06	147.9±4.9	293.0±7.2
6.	2.23±0.02	0.89±0.01	0.67±0.06	0.90±0.02	291.3±5.2	382.9±8.2
7	1.85±0.01	0.69±0.01	0.69±0.07	1.07±0.02	236.9±4.3	307.5±7.6
8.	1.78±0.01	1.20±0.02	0.67±0.05	0.73±0.03	326.7±7.5	328.7±4.5
9.	1.73±0.02	1.22±0.05	0.81±0.06	0.87±0.03	317.2±6.5	321.7±5.2

The changes in colour, occurring primarily due to oxidation processes (oxidation of phenols, degradation of chlorophyll and non-enzymatic browning due to the reaction of oxidised lipids with proteins), can be observed by evaluation algorithm describing the colour intensity of digitalised images. This method is more suitable for colour analysis of inhomogeneous food products than spot colorimeters, analyzing a very small surface of the sample. The evaluation of colour and intensity of product lightness, during oxidation process, through colourgram analysis, showed that the relative colour lightness, Figure 5., in all samples with 0.5g/kg of ascorbic acid containing lactoferrin, decreased more quickly during the time, i.e. that the browning was more intensive. The samples containing lactic acid browned abruptly (treatments 8 and 9), after a period of 150 min, while the samples containing ascorbic acid (1g/kg) and citric acid (1.5g/kg), (treatments 6 and 7), in which it also came to a decrease in making of lipid hydroperoxides, Table 1.), proved to be least prone to oxidation.

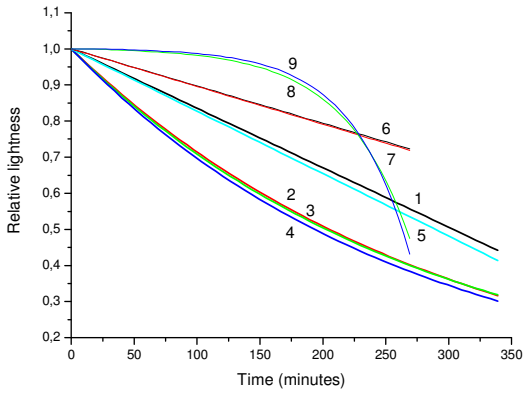

Fig. 5. Relative lightness of samples of basil-based ("*pesto*") spreads during oxidation on day 1st (min). The effect of 9 treatments with ascorbic, citric, lactic acid, NaCl and lactoferrin on browning. Treatments 1-9 as presented in Table 1. The SD for each treatment was: (No 1., 0.16521; No 2., 0.20045; No. 3, 0.19959; No. 4., 0.20421; No. 5., 0.17334; No. 6., 0.08201, No. 7., 0.08312; No. 9., 0.14284).

Conclusion

Lowering of the initial load of microorganisms in basil-based spreads, similar to Italian "*pesto*" sauce, was obtained through improvements of fresh basil leaves washing. The process of basil leaves washing, combining treatment with 2% H_2O_2 and mild temperature shock (50°C) significantly lowered the number of viable microorganisms (both total bacterial count and yeast and mould count). A combination of two temperature shocks, at 50°C and 90°C, led to the complete decrease in the number of viable microorganisms. Lowering of pH in minimally-processed "*pesto*" spreads decreases total bacterial count after 30 days of storage at 4°C. Low levels of lactoferrin, a natural metal-chelating protein, (80 and 400 mg/kg)

decreased lactic acid bacteria count at pH 5.2-5.3 and ascorbic acid concentration of 0.5 g/kg. Ascorbic and combination of ascorbic and citric acids inhibited lipid oxidation after 30 days of storage at 4°C. Lactoferrin, at the same levels that inhibit lactic acid bacteria count (80 and 400 mg/kg) had an antioxidative effect on the formation of lipid hydroperoxydes. Ascorbic and combination of ascorbic and citric acids inhibited browning of the product, (attributed to combined enzymatic and non-enzymatic oxidation), while lactoferrin slightly enhances browning in the presence of ascorbic acid (0.5 g/kg) at pH 5.2-5.3.

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PRIMENA METODE ANALIZE BOJE NA TAMNJENJE "*PESTO*" NAMAZA KONZERVIRANIH ORGANSKIM KISELINAMA I LAKTOFERINOM

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Rezime

Protein laktoferin, prirodni helator metala, potencijalno utiče na oksidaciju lipida, mikrobiologiju i tamnjenje (koje može poticati od enzimatske i neenzimatske oksidacije), što zavisi od uslova u proizvodu. Upoređen je uticaj kombinacije anti-oksidativnih i antimikrobnih agenasa na broj mikroorganizama, oksidaciju lipida i tamnjenje u "*pesto*" namazima od svežeg bosiljka. Tamnjenje je praćeno preko promene boje, primenom ocenjivanja boje preko višeparameterske analize. Laktoferin u koncentraciji od 80 i 400 mg/kg smanjuje akumulaciju primarnih oksidativnih produkata lipda (peroksidni broj) u izdvojenoj lipdnoj fazi, kao i broj *Lactobacillus* sp., posle mesec dana čuvanja na 4^{o}C. Kombinacija laktoferina i 0,5 g/kg askorbinske kiseline blago negativno utiče na oksidaciono indukciono vreme (OIT), određeno diferencijalnom skenirajućom kalorimetrijom (DSK) i brzinu tamnjenja proizvoda.