Obtaining nitrite from vegetables sources by fermentative process using nitrate-reducing bacteria *Sthaphylococcus carnosus* and *S. xylosus*

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Abstract
Sodium nitrate is an essential food additive widely used in cured meats. The increased unfavorable perceptions about chemicals in foods and their repercussions on health have positioned nitrites as undesirable compounds in meat products. Natural and organic ingredients have taken an important place within the consumer preferences. Here, obtaining nitrite from natural sources was achieved using nitrate-reducing bacteria *Staphylococcus carnosus* and *S. xylosus*. Pre-incubation strains into a celery-based medium allowed the stimulation of nitrate reductase activity. The increased values of substrate, the oxygen condition and pH influenced the nitrite generation significantly ($p<0.05$). The reduction rate from nitrate to nitrite was 50.85%, with a value of 320.2 mg L^{-1} of nitrite. This method presents characteristics comparable to those traditionally applied in the generation of conventional sodium nitrite leading the food industry to take advantage of fermentation processes to supply needs in meats and supplementary food products.

Keywords: sodium nitrite; nitrate; nitrite; nitrate-reductase; *Sthapylococcus carnosus*; *S. xylosus*.

Obtención de nitrito a partir de fuentes vegetales mediante proceso fermentativo usando bacterias nitrato-reductoras *Sthaphylococcus carnosus* y *S. xylosus*

Resumen
Nitrito de sodio es un aditivo alimentario ampliamente usado en carnes curadas. Percepciones negativas sobre el uso de agentes químicos en alimentos y sus repercusiones en la salud, han posicionado el nitrito como un compuesto no deseado en los productos cárnicos. Los ingredientes naturales y orgánicos han ocupado un lugar importante dentro de las preferencias de los consumidores. En este estudio, se obtuvo nitrito usando bacterias nitrato-reductoras *Sthaphylococcus carnosus* y *S. xylosus*. La pre-incubación de las cepas en un medio con base en apio, permitió la estimulación de la actividad nitrato reductasa. El aumento del sustrato, las condiciones de oxígeno y el pH, influenciaron significativamente la generación de nitrito ($p<0.05$). La tasa de reducción de nitrato a nitrito fue de 50.85%, con un valor de 320.3 mg L^{-1} de nitrito. Este método presenta características comparables a aquellos tradicionalmente aplicados para la generación del nitrito de sodio convencional.

Palabras clave: nitrito de sodio; nitrito; nitrato; nitrato-reductasa; *Sthaphylococcus carnosus; S. xylosus*.

1. Introduction

In the last few years consumer preferences have been aimed at demanding health-friendly, high quality, nutrient-rich natural products. Research on consumption behavior and market trends has provided evidence on individuals´ unfavorable perceptions of food additives, preservatives, and chemicals, in general [1].

Nitrites are among the most widely used compounds for the preservation of meat products. The food industry values

their use due to their great contribution to meats´ organoleptic characteristics and antimicrobial effects.

Within curing processes, nitrite is responsible for the red pigment production of cured meats, participating in the development of flavor and in slowing oxidative rancidity. In addition, it contributes to growth inhibition of pathogenic bacteria such us Clostridium botulinum [2]. Nevertheless, over the years great concerns have been expressed regarding the exposure of consumers to certain harmful products that may be formed in meat and meat products during and after curing [3]. The use of nitrites for meat curing became a major concern in the 1960s when it was discovered that nitrites have the potential to form carcinogenic nitrosamines when combined with secondary amines in a mildly acidic environment [4,5].

Although they have been considered to be negative in foods due to toxicological reasons, there is no strong epidemiological evidence for a correlation between nitrosamine formation and the incidence of gastrointestinal types of cancer. A recent meta-analysis of published papers (systematic literature searching of PubMed) provided a quantitative assessment of their relationships with gastric cancer, explaining that whereas human diet is a potentially modifiable exposure, it remains difficult to attribute the cancer etiology to a single nutrient [6].

In addition to nitrite and nitrate's essential functions such as food additives, those compounds have caused a deep impact on many physiological effects. Dietary consumption of nitrate has been demonstrated in clinical studies to have numerous health benefits, especially related to improving cardiovascular function and blood pressure [7]. Likewise, potential effects have been associated to the inorganic nitrate (NO_3-) inclusion on nutritional supplementation products in order to enhance muscle efficiency, tolerance and exercise resistance [8,9].

As we have seen, those compounds harbor relevant effects in different contexts. In the sense of the decreased consumer intentions toward conventionally produced and processed foods, studies have been conducted to evaluate new alternatives and develop innovative products that may support the essential nitrite and nitrate functions. In order to effectively respond to modern consumer needs, the food industry has focused research towards the inclusion of natural and organic ingredients that would maintain the quality and microbiological safety of the product. Natural curing processes and antimicrobial natural sources are examples of actual trends, decreasing components and chemical additives in meat products [10]. Nitrates/nitrites can be indirectly added to meat products without losing the common sensory effects of traditionally cured ones. Ingredients that naturally contain nitrates reflect the current trends [11].

Vegetables offer a great potential for introducing nitrates/nitrites in foods [12]. Examples of them are the families of Brassicaceae (rocket, radish, mustard), Chenopodiaceae (spinach, beets, spinach) Amarantaceae, Asteraceae (lettuce) and Apiaceae (celery, parsley) [12]. The broad interest highlighted on the organic and natural products intake have positioned those natural sources as potential alternatives to the replacement or the reduction of synthetic nitrates and/or nitrites. Here, the reduction of nitrate to nitrite

is a key step. This reaction may be represented by the use of chemistry-reducing agents, enzymatic processes, or by microorganism nitrate-reducers. Obtaining nitrate as substrate for nitrite generation through the action of nitrite-reducing bacteria, recognized as safe, represents a promising approach. The coagulase-negative staphylococci group (CNS) are well known, not only for presenting the enzymatic repertoire for this process, but for its compatibility with foodstuffs [13,14].

Considering the biological variability and metabolic behavior of the nitrate-reducing bacteria, it results important to study the appropriated conditions of nitrate-reductase activation. The expression of this enzyme in staphylococci, for example, may be induced by anaerobic growth under the nitrate presence, with the maximum level of activity during the exponential growth phase [15].

In line with this, the present study aimed to evaluate the nitrite generation from natural sources by fermentative process using nitrate-reducing bacteria. This alternative would allow enlarging new processed meat and supplementary food products with added natural compounds and a reduced level of nitrite to increase.

2. Methods

2.1. Nitrate extractions

Vegetable samples (broccoli and celery) were acquired from greengroceries of Medellin, Colombia. After transmission of the samples to the laboratory, they were washed with distilled water. Initially, different extraction methods were tested. 1) Disinfection/Maceration, 2) Scalding and 3) Maceration/autoclaving. They all were green extractions, based on the use of water, heat and mechanical disruptions in order to allow the derived product to be applied on foods.

For the first method, similar vegetables were mixed together and disinfected under submersion into ethanol 70% for exposition intervals of 5-10 minutes. Then, the samples were washed at least three times with sterile distilled water and led to dry at laboratory temperature for subsequent blender-based maceration. The scalding was applied to the same type of washed vegetables using a ratio water: vegetal of 12:1. This experimentation was carried out by using flasks subjected to constant agitation at 80°C for 15 min. Finally, the third method consisted in the submission of previous washed vegetables to blender-based macerations and following sterilization in autoclave at 121°C, 15 psi for 20 min.

2.2. Nitrate-reducing strains

Lyophilized strains Bactoferm C-P-77-S and S-SX composed by Staphyloccous carnous, Staphylococcus xylosus and Lactobacilus pentosus were used (Chr Hansen Hansen Inc., Milwaukee, Wis., U.S.A). A qualitative adaptive process was performed in order make conditions favorable for the microorganism tolerance to the new vegetable-based conditions of the fermentable media. Two types of culture media were assayed, a commercial Brain

heart infusion Broth (BHI OxoidTM) (37g/L) and a celery-based homemade broth (10% w/v) supplemented with yeast extract (5 g/L) and glucose (1 g/L). The culture media were autoclaved at 121°C, 15 psi, for 20 min.

A hydration/activation step of the strains was carried out into the prepared broths at 37°C for 24h. Succeeding the activations, the strains were cultured in selective agar plates with the purpose to obtain isolated colony forming units (CFU). Here, the Mannitol Salt agar (MSA) was used to isolate the Stahphylococcus from L. pentosus, considering that only the Sthaphylococcus strains have the enzymatic potential to reduce nitrate to nitrite. Once, obtained the characteristic morphology of the target microorganisms S. carnosus and S. xylosus, those were transferred to plate count agar (PCA) and incubated at 37°C for 24h. Finally, the colonies were used to prepare the inoculums (the Celery-activated and the BHI-activated) adjusted to a Macfarland pattern of 2.0 ($6x10^8$ CFU/mL). The purity of inoculums was verified by the observation of typical coccus gram positives by Gram staining.

2.3. Evaluation of fermentation conditions

Different fermentation processes were performed to look for the best conditions for an appropriated *nitrate-reductase* activity stimulation. Among them, the use of pre-adapted inoculums, the incubation under different oxygen conditions, pH values, fermentable culture supplementations, and the use of dried or fresh vegetal material were considered. Then, combinations of above mentioned conditions were examined. Two experimental repetitions and two technique repetitions were carried out for each condition. The use of synthetic sodium nitrate was used as control of fermentable substrate source.

2.3.1. Strain's pre-adaptation step and conditions of incubation

Initially, the inoculums activated either using the celery-based homemade or the BHI broth were used separately to evaluate the effects of the strain's pre-adaptation step to the fermentable vegetal conditions over the *nitrate-reductase* activity. Since, the blended fermentable media submitted to autoclaving process (121°C, 15 psi, for 20 min) showed to favor the nitrate extraction, no application of preceding extraction methods was needed.

Considering that the presence of oxygen may be also an influential factor on the stimulation of the nitrate and nitrite reductase activity of *S. carnosus* and *S xylosus,* the fermentation process was carried out under both aerobic and anaerobic conditions. The anaerobic atmosphere was achieved by pumping nitrogen gas into the Erlenmeyer flasks for 3 minutes (dimensions 140 x 83 mm, height x widest diameter) and sealing it with rubber stopples. Anaerobic indicators (BD, BBL Dry anaerobic Indicator strips, Becton Dickinson and Company) were introduced in each flask. The experiment was incubated in a covered shaker water bath (SW23, Julabo) at 30°C, 150 rpm for 24h.

Table 1.
Fermentable culture media compositions.

Component	Fermentable Culture media		
	1	2	3
Celery powder (25 g/L)/ Fresh celery (100g/L)	5 /20 g	5 /20 g	5 /20 g
Yeast extract (5 g/L)	1 g	1 g	1 g
Dextrose (1 g/L)	0.2 g	0.2 g	0.2 g
NaCl (2 g/L)	0.4 g	0.4 g	___
K_2HPO_4 (13 g/L)	2.6 g	2.6 g	___
Brain heart infusion- BHI (10 g/L)	2 g	___	___
Potassium caseinate (10 g/L)	___	2 g	___
Distilled water (mL)	200	200	200
pH value	9.0	9.0	9.0

Source: The Authors.

2.3.2. Influence of pH values

Once found the anaerobic atmosphere and strains preadaptation as determining features to prompt the bacteria enzymatic action, variations in the pH values of the fermentable media were assessed. For this purpose, only the base of fermentable media, mainly composed by celery 10% w/v, yeast extract 5 g/L, and dextrose 1g/L were used. The pH values ranged from 7 to 10. The inoculum adapted on the nitrate-rich celery-based broth (10% v/v) was aseptically added to the fermentable media under a biological laminal flow chamber. The media without pH adjusting was took like negative control. The experimentations were incubated in a covered shaker water bath (SW23, Julabo) at 30°C, 150 rpm for 24h. Experimentations were performed in duplicate.

2.3.3. Supplementation of celery-based fermentable media

The fermentable media was mainly composed of 10% w/v celery, yeast extract 5 g/L, and dextrose 1 g/L. But, they were either enriched with BHI 10 g/L, potassium caseinate 10 g/L, NaCl 2 g/L, K_2HPO_4 13 g/L. The addition of those compounds to the media varied as shown in the Table 1. Consequently, three different media compositions were evaluated.

Prior studies showed an increased nitrite concentration in the fermentable media during the first hours of incubation. In this sense, here, unlike the previous fermentations, the duplicated flasks were incubated until 6 hours under the same aerobic and anaerobic conditions as above explained.

2.3.4. Use of dried or fresh vegetal

The nitrate level in vegetable products is highly variable. It depends on the biological properties of the vegetal culture, such as the plant size, soil composition, density growth, plant maturity, duration of growth, harvest time, nitrogen source, time and storage conditions [16]. In this study, we intended to standardize the fermentable medium using a laboratory-made normalized celery powder. Then, both the fresh celery based medium (10% w/v) and the celery powder based medium (2.5% w/v) were fermented. The media composition was BHI 10 g/L, potassium caseinate 10 g/L, NaCl 2 g/L, K_2HPO_4 13 g/L and 10% inoculum v/v. The

fermentations were carried out at 30°C, in constant agitation (150 rpm) for 6h, under anaerobiosis.

2.4. Analysis of nitrate/nitrite concentrations

2.4.1. Samples preparation

As nitrates were extracted by the autoclaving process of each celery-based fermentable media, aliquots were taken before and after fermentation to determine nitrate/nitrite contents. Samples were centrifuged at 3000 x g for 15 min (Botofix 32a, Hettich Zentrifugen); the supernatants collected were filter-purified (0.2μm Syringe filter, membrane NYL) to eliminate bacterial cells, and were then, transferred to 1 mL dark micro-vials. All samples were immediately analyzed within 1 hr. after sample preparation.

An Agilent 1260 infinity - high performance liquid chromatography system, equipped with photodiode-array UV detector was utilized in this study. A C18 column (5 μm, 250 4.6 mm I. D.) was employed for separation.

2.4.2. Preparation of mobile phase and standard solutions

Aqueous solutions consisted of Potassium dihydrogen phosphate (KH_2PO_4 Merck 99.5 per cent pure) and Sodium hypophosphite (Na_2HPO_2 Merck Darmstalt, Germany 99% Purity), prepared in deionized Milli-Q water (Millipore). The adjustment of pH value to 3.0 was done by adding phosphoric acid.

Standard solutions containing 200 mg L^{-1} of sodium nitrate and sodium nitrite were prepared, adjusted pH to 6.15. The pH values were monitored using pH-meter HandyLab (Schott instruments) and Analytics pH electrode Blueline. The solutions were freshly prepared for every running HPLC chromatograms. The calculated standard curve and correlation coefficients was indicative of the linearity within the tested range of concentrations.

2.4.3. HPLC analysis

In this study, the evaluation of the nitrate-reductase activity of *S. carnosus* and *S. xylosus* viable cells was based on the measurements of residual nitrate and the generated nitrite in the fermented celery-composed media. The mobile phase solution was allowed to pass through the HPLC column until a stable baseline signal was equilibrated. The flow rate was 1.0 mL/min and the detecting UV wavelength was 214 nm. When the injections of the nitrate/nitrite standard solutions gave stable and reproducible retention times and peak areas, each filtered sample was then injected for analysis. The injection volume was 5.0 μL. The peaks of the samples were identified by comparison to the fitting peaks of the standards. When the curve of the peak areas was larger than that of the maximum amount from the standard curve, the test solution was diluted to appropriate concentrations. The amounts of nitrate and nitrite in the test solution were calculated from the peak areas by using linear regression equations of nitrate and nitrite standard curves. At the end of the analysis, the HPLC column was washed.

2.5. Statistical analysis

The statistical significance of the differences in the measured parameters in each fermentable medium type were tested by the analysis of variance (ANOVA) test. Here, the generation rate of nitrite was compared. A *student-t* test was further performed to compare the mean of nitrate/nitrite levels, variations in the vegetal type and pH values. The results shown in the figures are the mean values ± SD. Mean values are significantly different at $P < 0.05$, as indicated by asterisks (*) or letters in the figures. Graphpad Prism 7.0 software was used.

3. Results and discussion

The concerns about the use of nitrites are extensively linked to the formation of toxicological compounds in cured meat products. However, less is known about the traditional methods used for obtaining synthetic nitrate/nitrite at industrial level, which may embrace process highly intervened by chemical agents. Here, we presented an alternative approach to obtain nitrite from natural sources attuned with the current consumers preferences. Natural nitrite differs from synthetic nitrite in the way that they are produced. Synthetic nitrites are linked to chemical processes, as are typically created by the use of C (carbon), Fe (iron) or Pb (lead) for nitrate reduction at elevated temperatures. The industrial manufactured $NaNO_2$ has been described as a result of bubbling a mixture, comprising NO and NO_2 in a hydroxide solution by alkali metal nitrites [17].

A variety of vegetables have shown high nitrate contents. In the present study, we evaluated food-compatible nitrate extractions of two vegetables with textural differences, Broccoli and Celery. The application of three extraction techniques resulted in obtaining nitrate levels from 795.87 mg L^{-1} to 1045.62 mg L^{-1} for celery samples and from 189.48 mg L^{-1} to 261.74 mg L^{-1} for broccoli. The nitrate quantities exhibited variations according to the applied extraction method and vegetable type. The application of maceration/autoclaving technique registered the high nitrate value for both celery and broccoli, with 1045.62 mg L^{-1} and 261.74 mg L^{-1} respectively. Significant differences were found when compared to the other applied techniques (p<0, 05). On the other hand, scalding and disinfection/maceration procedures showed similar extracting abilities for both vegetables, resulting in 852,48 and 795,87 mg L^{-1} in the case of celery and 199.13 and 189.48 mg L^{-1} nitrate for broccoli. Those nitrate levels are comparable with previous studies. Iammarino *et al.* (2004) reported extraction values from 35.0-1800 mg L^{-1} of nitrate using a homogenization-based method at 80°C for 5 min, and values so high as 2978.1 and 2291.8 mg L^{-1} of nitrate obtained from frizzed and fresh spinaches [18]. The results here exposed reveal the importance of heat application during the extraction phase. Despite variations on the design of the techniques reported in several studies, including differences in the vegetable type and time treatment, all have as common element the use of hot water preceded by proper vegetable homogenization [19, 20]. It was perceived that when extractions were performed at bench temperature or lower than 80°C (the case of

disinfection/maceration and scalding), the obtained nitrate concentration was reduced. Consequently, the maceration/autoclaving was kept as a combined method of nitrite extraction and sterilizations, which at time, guarantees an aseptic media for the strains metabolic activity.

Since the celery vegetal showed higher substrate levels, and exhibited a reduced pigmentation in the obtained juice, it was chosen as the appropriate source to further fermentations. Nitrate reduction to nitrite was improved when used the inoculum pre-adapted on the nitrate-rich celery-based broth. The HPLC chromatograms (Fig. 1) showed an elevated nitrite level of 392.035 mg L^{-1} obtained from celery-based fermented media for 24h and did not registered nitrite levels in the synthetic nitrate-based fermentable control media. While the strains were pre-incubated in the commercial BHI broth, the nitrite obtained from the nitrate-rich celery-based broth was diminished, resulting in 49.497 mg L^{-1} of nitrate.

In general, there was no generated nitrite, when using the strains not adapted; a reduced nitrate was found after 24h of the anaerobic fermentation process, compared to the initial content. Here, the substrate level fell 26.6% compared to the non fermentable media. All the experimentations carried out under aerobic conditions did not show nitrite generation. On the contrary, the celery-based fermentations submitted under anaerobic atmosphere presented a mean nitrite level of 392.035 mg L^{-1} ± 5.045, indicating a 73,27% of reduction from nitrate to nitrate (535 mg L^{-1} initial nitrate and 392.035 mg L^{-1} final nitrite).

The earlier strains exposition to the vegetal material favor the enzymatic activity; therefore, all accumulations of nitrite was the result of the action of adapted viable cells. The induction of the nitrate-reductase activity on strain of *S. carnosus* have been reported elevated when the substrate was added to the growth culture medium. The nitrate-reducing rate was 8 to 10 times higher in anoxic conditions [15]. These notions are clearly supported by the results here obtained considering that the pre-adaptation step to the fermentable vegetable and the anaerobic atmosphere were key factors in reflecting improved nitrate-reducing rate. It was noticed that the stimulation of nitrate-reductase activity was proportional to the increased nitrate concentration in the fermentable media, suggesting that nitrate reduction is not attached to the sources type, but to enough substrate availability. In this context, authors have explained that the NO_2^- liberation to the media by the bacteria take place if the NO_3^- level in the media is higher than 5 mM [21]. This allows the bacteria using the nitrate produced intracellulary as nitrogen source for the cellular metabolism, and hence, it is not secreted to the external media.

During respiration, the bacteria *S. carnous* and *S. xylosus* use nitrate as electron receptor when the oxygen is not available. Thus, the nitrate reduction is coupled to the generation of a driving force of protons which is directly taken as energy source [15]. Associated mechanisms to the activation of the nitrate-reductase activity still need to be elucidated. However, prior research has reported a regulator gene named *NreABC*, in charge of the detection of anoxic conditions, as well as, the nitrate presence, resulting in the nitrate-reducing activation system on the bacteria [22].
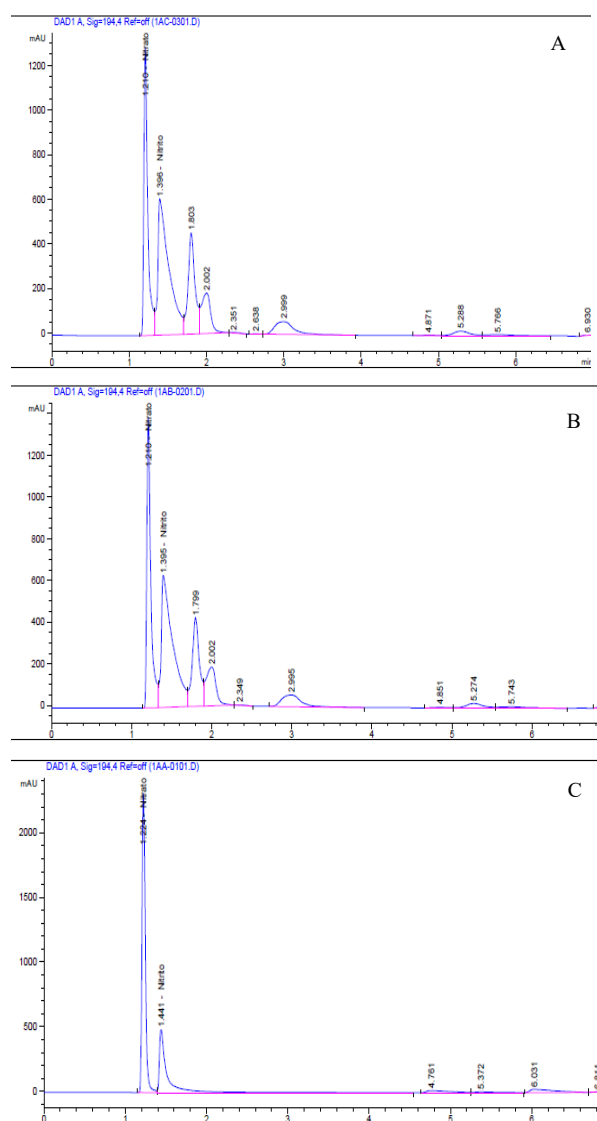

Figure 1. HPLC chromatograms of fermented samples for 24h. A-B) Nitrate-rich celery-based media C) Nitrate/nitrite sodium standards.
Source: The Authors.

The first goal of this study was to find out the appropriate conditions of the fermentation process that guaranteed at time, an elevated staphylococci enzymatic action. Regarding to the evaluated pH values, better nitrate-reduction was registered at pH between 8.0 to 9.0, with 255.25 and 281.39 mg L^{-1} of nitrite respectively (Table 2.). The ordinary one-way ANOVA analysis showed significant difference between the treatments $p < 0.05$. The media which pH value was not adjusted, presented acid pH ranged between 4.0 to 5.0. Multiple comparisons test against unadjusted experimentations were statistical significant $p < 0.05$. Once compared by *student-t* test the two higher registered levels of nitrite (pH 8.0 and 9.0), a difference was observed (p 0.0089). It's possible that the pH decrease during the fermentation was the result of glucose degradation, and hence, the appearance of derived metabolites such as acetate and pyruvate [21]. Here, the use of buffers in the fermentable media are

Table 2.
Peaks area of nitrate and nitrite obtained on running chromatograms from samples at different pHs.

pH	NO₃			NO₂		
	Ret. time (min)	Area	mg L⁻¹ nitrate	Ret. time (min)	Area	mg L⁻¹ nitrite
CT	1.359	10697.1	306.61	1.774	414.2	36.84
7	1.352	10136.9	290.55	1.778	2152.9	191.4*
8	1.354	11724.9	336.07	1.725	2869.9	255.2*a
9	1.355	11837.9	339.31	1.727	3163.8	281.3*b
10	1.359	10172.0	291.56	1.771	845.3	75.18*
SD	1.358	6977.66	200	1.721	2248.7	200

*Values significantly differents compared to unadjusted pH control (CT), SD (standard) $p<0.05$. Tretment with distint letters are statisticaly different by unpaired t-test $p<0.05$.
Source: The Authors.

important to mantain a balanced pH and support the growth rate of the Staphylococci strains, which have been reported inhibited at pH 4.5 [23].

The glucose, yeast extract, and the presence of celery-derived nitrate as fermentable substrate played an important role as electron donor (glucose) and electron acceptor (nitrate). When they are added to the fermentable media under anoxic atmosphere, the biomass production and the nitrate reduction may increase. This suggest that there was a diminution on the energetic efficiency of the bacterial anaerobic respiration when incubated under oxygenic condition, and then the glucose could be catabolized to other metabolites [24]. The fermentable media composition found adequate to further fermentation process was then achieved (see Table 1). Its components were fresh celery, yeast extract, dextrose, BHI and NaCl and buffer agents. Despite the use of celery powder allowed to standardize the vegetal source, the addition of 2.5% w/v to the fermentable medium showed decreased solubility, affecting the agitation during the fermentation process. Here, the fermentation kinetic for 6 hours showed better nitrate-reduction rate, registering the higher nitrate-*reductase* activity between 4-5 hours (Fig. 2).

The enzyme nitrate-reductase on *S. carnosus* have been described to be attached to the membrane and involved on the conservation of the respiratory energy [15]. The maximum profile expression has been reported during the exponential phase. On the other hand, the enzyme nitrite-reductase may be found in a cytosolic fraction. The action of this enzyme only is activated under anaerobic conditions and is induced by the presence of nitrate or nitrite in the of growth media [25]. Here, the indirect regulation of those enzymes may be achieved by modulating the concentrations of the subtracts in the fermentable media.

S. carnosus and *S. xylosus* may reduce nitrate to ammonia in two steps: First, the nitrate is absorbed and reduced to nitrite, and the nitrite is excreted later. Second, after the depletion of the nitrate in the fermentable media, the accumulated nitrite is imported to the interior of the cell, reduced to ammonia, and this one, it is accumulated again in media [22,26].

It is possible that the reduced nitrite level in most of the fermentations that took 24 hours was obtained as result of the catabolism of the produced nitrite, once the nutrient and other
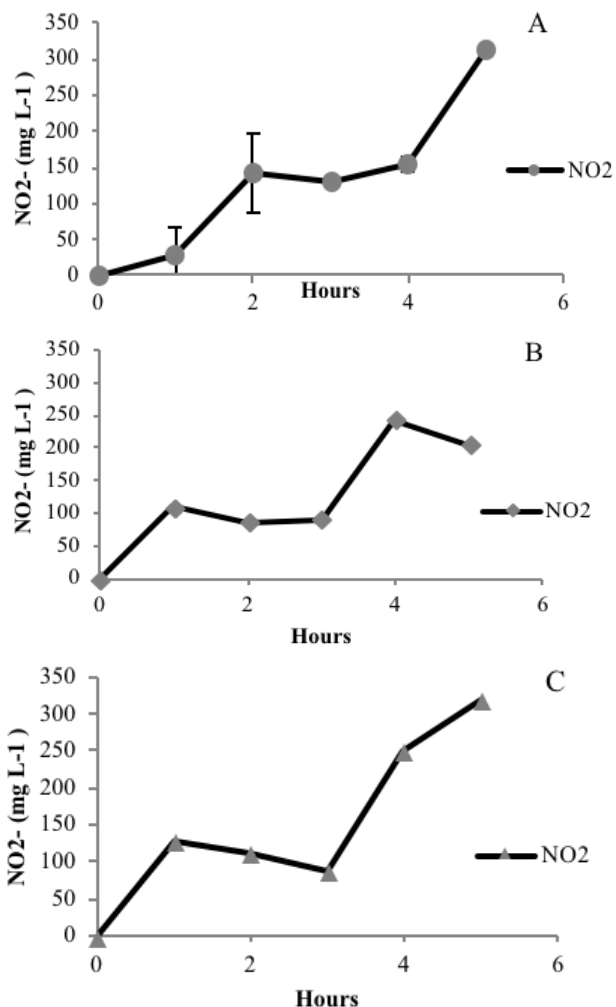

Figure 2. Kinects of fermentation process using A) Fresh celery, B) Celery powder and C) Synthetic nitrate as substrate source.
Source: The Authors.

substrates could be also diminished. Consistent with the above results, short periods of fermentations were performed to catch earlier levels of produced nitrite and avoid the simultaneous degradation of the same secreted nitrite from the media.

Conditions found to favor *nitrate-reductase* activity of *S. carnosus* and *S. xylosus* consisted of a chain of steps, starting with the pre-adaptation strains to a nitrate-rich celery-based medium, followed by the composition of fermentable media, then the adjustment of pH value was adjusted to 9.0, after which the anoxic condition of incubation was induced. Finally, the medium was exposed to agitation. Additionally, no less important, was the time of fermentation (4 h). After those characteristics favorable for generating nitrite were identified, additional experimentations were performed. Consequently, after 4h of fermentative process of celery-based media, a nitrate-reduction rate of 50.85% was observed, passing from 629.42 to 309.22 mg L⁻¹ of nitrate and generating 302.2 mg L⁻¹ of nitrite. As seen, the entire procedure for obtaining nitrites/nitrate is advantageous to apply to the meat industry. Within the meat industry,

comparable findings have been reported regarding to the residual nitrite contents in manufactured meat products. Prior studies have demonstrated that the use of sodium nitrite in cooked ham results in a residual nitrite level of around 90 mg Kg^{-1} compared to the use of celery juice as a source of nitrite, which results in a residual level of nitrite between 30 mg Kg^{-1} and 60 mg K^{-1} [27].

4. Conclusions

Nitrate from vegetable sources were obtained in the present study without using chemical agents. The parameters evaluated led to select processes as simple as the mechanical maceration and sterilization by autoclave, like adequate technique to extract nitrate. Key parameters were identified to run fermentative process with better yield on staphylococci nitrate reduction. Here, the availability of high subtrate level as crucial element regulator on the *nitrate-reductase* activity was underlined, as well as the previous adaptation of the bacteria to a nitrate-rich selective environment was identified.

The use of enzymatical agents and nitrate-reducing microorganisms, generally recognized as safe (GRAS), promise to be better alternatives and to reduce the use of chemical compounds in the production of nitrite. These non-toxic processes, are useful, not only to for the meat industry (as an agent of curing), but also for the formulation of food supplements, with emphasis in sports performance, and other physiological endeavors, which recently have been associated with benefiting from nitrate consumption. Consumer preferences towards natural products is leading the food industry to seek natural options. The processes outlined in this paper can help supply the market needs for natural products.

Acknowledgements

Bibliography

[1] Lee, H.-J. and Yun, Z.-S., Consumers' perceptions of organic food attributes and cognitive and affective attitudes as determinants of their purchase intentions toward organic food, Food Qual. Prefer., 39, pp. 259-267, 2015. DOI: 10.1016/j.foodqual.2014.06.002

[2] Sindelar, J.J. and Milkowski, A.L., Sodium nitrite in processed meat and poultry meats: a review of curing and examining the risk/benefit of its use, Am. MEAT Sci. Assoc., 3, pp. 1-14, 2011.

[3] Alahakoon, A.U., Jayasena, D.D., Ramachandra, S. and Jo, C., Alternatives to nitrite in processed meat: up to date, Trends Food Sci. Technol., 45, pp. 37-49, 2015. DOI: 10.1016/j.tifs.2015.05.008

[4] Usinger, E.L., Larson, E.M., Niebuhr, S.E., Fedler, C.A., Prusa, K.J., Dickson, J.S., Tarté, R. and Sebranek, J.G., Can supplemental nitrate in cured meats be used as a means of increasing residual and dietary nitrate and subsequent potential for physiological nitric oxide without affecting product properties?, Meat Sci., 121, pp. 324-332, 2016. DOI: 10.1016/j.meatsci.2016.06.022

[5] Butler, A., Nitrites and nitrates in the human diet: carcinogens or beneficial hypotensive agents?, J. Ethnopharmacol., 167, pp. 105-107, 2015. DOI: 10.1016/j.jep.2014.09.040

[6] Song, P., Wu, L. and Guan, W., Dietary nitrates, nitrites, and nitrosamines intake and the risk of gastric cancer: a meta-analysis, Nutrients, 7, pp. 9872-9895, 2015. DOI: 10.3390/nu7125505.

[7] Bedale, W., Sindelar, J.J. and Milkowski, A.L., Dietary nitrate and nitrite: benefits, risks, and evolving perceptions, Meat Sci., 120, pp. 85-92, 2016. DOI: 10.1016/j.meatsci.2016.03.009

[8] Wylie, L.J., Mohr, M., Krustrup, P., Jackman, S.R., Ermıdis, G., Kelly, J., Black, M.I., Bailey, S.J., Vanhatalo, A. and Jones, A.M., Dietary nitrate supplementation improves team sport-specific intense intermittent exercise performance, Eur. J. Appl. Physiol., 113, pp. 1673-1684, 2013. DOI: 10.1007/s00421-013-2589-8

[9] Hoon, M.W., Johnson, N.A., Chapman, P.G. and Burke, L.M., The effect of nitrate supplementation on exercise performance in healthy individuals: a systematic review and meta-analysis, Int. J. Sport Nutr. Exerc. Metab., 23, pp. 522-532, 2013.

[10] Sebranek, J. and Bacus, J., Natural and organic cured meat products: regulatory, manufacturing, marketing, quality and safety issues, Am. Meat Sci. Assoc., 1, pp. 1-15, 2007.

[11] Sebranek, J.G., Jackson-Davis, A.L., Myers, K.L. and Lavieri, N.A., Beyond celery and starter culture: advances in natural/organic curing processes in the United States, Meat Sci., 92, pp. 267-273, 2012. DOI: 10.1016/j.meatsci.2012.03.002

[12] Marco, A., Navarro, J.L. and Flores, M., The influence of nitrite and nitrate on microbial, chemical and sensory parameters of slow dry fermented sausage, Meat Sci., 73, pp. 660-673, 2006. DOI: 10.1016/j.meatsci.2006.03.011

[13] Sarabia, L., Efecto del uso de BactofermTM LHP (*Pediococcus acidilactici* & *Pediococcus pentosaceus*), BactofermTM F-RM-52 (*Lactobacillus curvatus* & *Staphylococcus carnosus*), BactofermTM F-LC (*Pediococcus acidilactici, Lactobacillus curvatus* and *Staphylococcus xylosus*) y Cultivo lácteo SLB 953 (*Lactobacillus bulgaricus* & *Streptococcus thermophilus*) en la elaboración de chorizo (tipo Ambateño) madurado, Tesis, Universidad Técnica de Ambato, Ecudor [en línea]. 2011. [acceso: enenro13 de 2015]. Disponible en: http://repositorio.uta.edu.ec/handle/123456789/3076

[14] Rosenstein, R. and Götz, F., What distinguishes highly pathogenic staphylococci from medium- and non-pathogenic? in: Dobrindt, U., Hacker, J.H. and Svanborg, C., (Eds.), Pathog. Commensalism, Springer, Berlin, Heidelberg, [online]. 2012, pp. 33-89. [accessed: April 8, 2016]. Available at: http://link.springer.com/10.1007/82_2012_286 DOI: 10.1007/82_2012_286

[15] Neubauer, H. and Götz, F., Physiology and interaction of nitrate and nitrite reduction in staphylococcus carnosus, Journal of Bacteriology, 178, pp. 2005-2009, 1996. DOI: 10.1128/jb.178.7.2005-2009.1996.

[16] Tamme, T., Reinik, M. and Roasto, M., Nitrates and nitrites in vegetables: occurrence and health risks, Bioact. Foods Promot. Health (Fruits Veg.), pp. 307-321, 2010. DOI: 10.1016/B978-0-12-374628-3.00021-9

[17] Djeri, N., Evaluation of Veg Stable TM 504 celery juice powder for use in processed meat and poultry as a nitrite replacer, Dissertations & Theses, PhD., University of Florida, [online]. 2010. [(accessed: January 13, 2015]. Available at: http://search.proquest.com/docview/899719470).

[18] Iammarino, M., Di Taranto, A. and Cristino, M., Monitoring of nitrites and nitrate levels in leafy vegetables (spinach and lettuce): a contribution to risk assessment, J. Sci. Food Agric., 94, pp. 773-778, 2014. DOI: 10.1002/jsfa.6439

[19] Correia, M., Barroso, Â., Barroso, M.F., Soares, D., Oliveira, M.B.P.P. and Delerue-Matos, C., Contribution of different vegetable types to exogenous nitrate and nitrite exposure, Food Chem., 120, pp. 960-966, 2010. DOI: 10.1016/j.foodchem.2009.11.030.

[20] Gaiser, M., Rathjen, A. and Spiess, W.E.L., Nitrate extraction during blanching of spinach, LWT - Food Sci. Technol., 30, pp. 432-435, 1997. DOI: 10.1006/fstl.1996.0200

[21] Martínez, R., Fisiología de la asimilación de nitrógeno en "Haloferax mediterranei". Purificación y caracterización de Nitrato y Nitrito reductasas asimilativas, Tesis Doctoral, Universidad de Alicante, España,2003.

[22] Niemann, V., Koch-Singenstreu, M., Neu, A., Nilkens, S., Götz, F., Unden, G. and Stehle, T., The NreA protein functions as a Nitrate receptor in the Staphylococcal Nitrate regulation system, J. Mol. Biol., 426, pp. 1539-1553, 2014. DOI: 10.1016/j.jmb.2013.12.026

[23] Dilsen, S., Paul, W., Herforth, D., Sandgathe, A., Altenbach-Rehm, Freudl, J.R., Wandrey, C. and Weuster-Botz, D., Evaluation of

parallel operated small-scale bubble columns for microbial process development using Staphylococcus carnosus, J. Biotechnol., 88, pp. 77-84, 2001. DOI: 10.1016/S0168-1656(01)00265-6

[24] Casaburi, A., Blaiotta, G., Mauriello, G., Pepe, O. and Villani, F., Technological activities of Staphylococcus carnosus and Staphylococcus simulans strains isolated from fermented sausages, Meat Sci., 71, pp. 643-650, 2005. DOI: 10.1016/j.meatsci.2005.05.008

[25] Gøtterup, J., Olsen, K., Knöchel, S., Tjener, K., Stahnke, L.H. and Møller, J.K.S., Relationship between nitrate/nitrite reductase activities in meat associated staphylococci and nitrosylmyoglobin formation in a cured meat model system, Int. J. Food Microbiol., 120, pp. 303-310, 2007. DOI: 10.1016/j.ijfoodmicro.2007.08.034

[26] Fedtke, I., Kamps, A., Krismer, B. and Gotz, F., The Nitrate reductase and Nitrite reductase operons and the narT gene of Staphylococcus carnosus are positively controlled by the novel two-component system NreBC, J. Bacteriol., 184, pp. 6624-6634, 2002. DOI: 10.1128/JB.184.23.6624-6634.2002

[27] Sebranek, J.G. and Bacus, J.N., Cured meat products without direct addition of nitrate or nitrite: what are the issues?, Meat Sci., 77, pp. 136-147, 2007. DOI: 10.1016/j.meatsci.2007.03.025

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