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BIOINPUTS: MICROBIOLOGICAL ANALYSIS EM SAMPLES OF LETTUCE (*Lactuca sativa L.*) COMMERCIALIZED IN THE MUNICIPAL MARKET OF ANÁPOLIS-BRAZIL

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ABSTRACT

The lettuce (*Lactuca sativa L.*) is among others the most consumed in natura in Brazil, it has nutritional values indispensable to human health, having a low content of calories, vitamins A, B and C, fibers and minerals (phosphorus, iron, sodium and potassium). According to the World Health Organization (WHO), most Foodborne Diseases are transmitted through theconsumption of food or contaminated water. Considering the risks of the predominant pathogens in lettuce, this work aimed to find and identify possible microorganisms in lettuce samples. The development of the work carried out the following steps: firstly, the analysis was performed using the Flow Injection Analysis (FIA) system with spectrophotometric detection, to verify the presence of microorganisms in solutions obtained with lettuce samples. After the detection of microorganisms in the referred solutions, microbiological analyzes were performed to identify them. In these analyzes the bacteria Escherichia coli and Staphylococcus aureus were identified.

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INTRODUCTION

Vegetables are foods that have essential nutritional principles for human health: carbohydrates, proteins, minerals, vitamin A, B and C, as well as fibers that help bowel movements as they form a residue that helps the bowel to function properly (Ferreira, *et al*., 2002; Correia, 2013). However, the lack of hygiene and the precarious sanitary conditions can favour the development of parasitic infections according to data from the WHO, most Foodborne Diseases are caused by the consumption of water (Sapers & Simmons, 1998; Srebernich *et al*., 2008). The most common symptoms presented are: acute diarrhea, muscle aches, fever, and vomiting. They presuppose that one in ten people become ill due to these contaminations, and when there is no adequate treatment it can lead to death. Regarding these issues, it is estimated that 420,000 deaths occur each year (Oliveira, 2016). Microorganisms can host in vegetables and greens through the production and handling chain, ranging from planting to final preparation for consumption (Queiroz *et al*., 2012; Suinaga *et al*., 2013; Carvalho & Silveira, 2014; Gimenez, 1994; Pacheco *et al*., 2002). Among the pathogens found in vegetables, total coliforms stand out, with *Escherichia coli* being the main representative of thermotolerant coliforms, considered by the Brazilian Ministry of Health as the most specific indicator of fecal contamination (Queiroz *et al*., 2012; Suinaga *et al*., 2013; Carvalho & Silveira, 2014; Gimenez, 1994). *Salmonella sp* is found in raw vegetables, so if the vegetable is consumed without a wash with sanitizing solution this contamination can cause epidemics. For this reason, it constitutes a relevant public health problem (Carvalho & Silveira, 2014; Gimenez, 1994). According to the International Commission for Microbiological Specifications for Foods (ICMSF), the bacterium *Staphylococcus aureus* also stands out as a pathogen found in food, but it is considered a small risk bacterium and does not lead to death or major discomfort in the person who contract (Srebernich *et al*., 2008; Oliveira, 2016). In order to improve the quality of vegetables against pathogens, prevention and control measures were sought at all stages of production, aiming at greater consumer safety, avoiding Foodborne Diseases (Queiroz *et al*., 2012; Suinaga *et al*., 2013; Carvalho & Silveira, 2014; Gimenez, 1994; Pacheco *et al*., 2002; Scherrer & Marcon, 2016; Santos, 2018).

MATERIAL AND METHODS

The development of the research was divided in stages according to the flowchart (Figure 1).

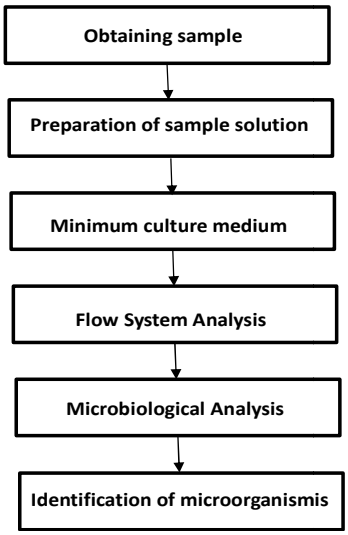

Figure 1. Flo: Stages of development of research activities

The experiments were carried out with the support of the Chemistry and Microbiology Laboratories, at the Exact and Technological Sciences Campus - Henrique Santillo of the State University of Goiás (CCET-UEG).

Obtaining samples: The lettuce samples (*Lactuca sativa* L.) were collected at the Municipal Market of Anápolis, in a vegetable stand of the main distribution center. Samples were collected during September 2018, with an interval of one week. They were adequately conditioned in plastic bags, hermetically sealed, taken to the microbiology laboratory of the State University of Goiás (CCET-UEG), where they were submitted to the process of preparation of solutions and microbiological analysis for the detection and identification of possible microorganisms.

Microbiological analysis: The leaves of the lettuce samples were selected, prioritizing the intermediate region between the outer and inner leaves of the vegetable. 10 g of the selected leaves were weighed, each leaf was washed individually, to remove the dirt (organic matter). Subsequently, it was washed with peptone water, generating a solution of the sample to be subjected to analysis. The samples were analyzed in serial dilutions: 10^0, 10^{-1}, 10^{-2}, 10^{-3}. 100 µL of each dilution was inoculated into the nutrient agar in petri dishes. Then the plates were incubated at 37 °C for 24 hours, to promote the proliferation of possible microorganisms, for counting and isolating them. For counting, he selected the petri dishes that contained between 30 and 300 cfu, (colony forming unit). All procedures were performed in duplicates.

Equipments and accessories: The continuous flow system built in the laboratories of CCET-UEG campus, whose analysis module is represented in figure 1, was constituted with a peristaltic pump, Milan, model 204, equipped with tubes of Tygon, to effect the pumping of the solutions , PTFE tubes with internal diameter of 0.8 mm, for channeling solutions, two solenoid three-way valves, Nresearch 225T031, Aloje, MA, USA; two timers, built in the laboratory, to connect the valves for a defined time; a gas-diffusing chamber, built in acrylic, composed of a teflon membrane, to allow separation of the carbon dioxide (CO_2) from the sample medium, a Spectrophotometer, Uv Vis, Kasuaki IL - 593, to monitor variations in the turbidity produced by the microorganisms on the sample solution. A water bath Solab, Model: SL 155, to maintain the temperature of the solution around 37°C, ideal condition for the proliferation of the inoculated microorganisms.

Description of the operation of the flow system used: The analysis module of the flow system used to detect microorganisms, as well as to monitor their proliferation, is shown in Figure 2.

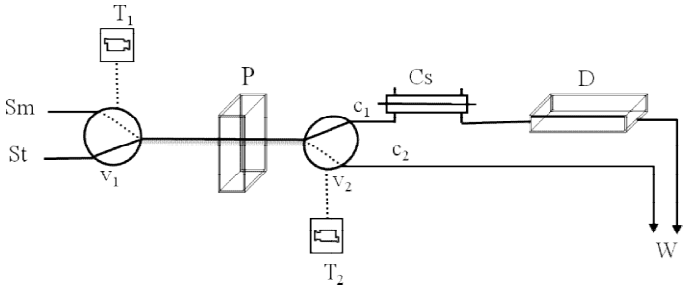

Figure 2. Flow system analysis module with spectrophotometric detection. Sm = sample; St = carrier solution (deionized H_2O); V_1 and V_2 = three-way solenoid valves; P = peristaltic pump; c_1 = path 1; c_2 =path 2; Cs = gas diffusion separation chamber; T_1 and T_2= semiautomatic timers; D = UV/Vis Spectrophotometer; Ws = discard

According to the analysis module (Figure 2), with the valves V1 and V2 being switched off, the carrier solution flows through the path c1 through the separation chamber Cd to the discard Ws. To insert the sample solution, the valve (V1), for a predetermined time interval,in each flow to delimit the volume of sample to be inserted in the analytical path of the flow system. After samplingV1 is switched off, flowing back the carrier solution take the sample solution through the gas diffusion chamber used to eliminate the CO_2 produced by the microorganisms in the sample solutions,thus avoiding instability of readings in the sample detector. The mixture of the sample solution with the solution flows through the c1 path to the discard through the detector cuvette, where an absorbance signal is generated, proportional to the turbidity produced as a function of the proliferation of the microorganisms in the sample solutions. The valve (V2) positioned on the analytical path was only switched on during exchange of sample solution, this is used to deflect sample solution residues, along the path c2 going into the discard, without passing through the detector. Thus c2 is coupled together with v1 for a suitable time interval to effect the exchange of sample solution and thereby speed up the analytical frequency. Then, V1 and V2 are switched off, flowing back to the carrier solution through c1. In this condition, a new sampling cycle can be started. As for semiautomatic timers, they are devices built in the laboratory, with a time scale ranging from 0.5 seconds to 5 minutes. These accessories allow one to turn on and off electronic components in the time range defined by the analyst. It consists of a key that once activated, turnsthe unit on and keeps it on for the period of time previously set, at the end of this period automatically turns it off. For this flow system, the timers were used to switch on and off three-way solenoid valves V1 and V2 for defined periods, according to the optimization conditions of the variables required for the chemical analysis. Sampling is done using timer T1 to switch V1. The switching period of V1 to delimit the volume of the sample solution must be previously defined as the optimization variable. This must be held constant to accurately reproduce the volume of sample to be inserted in the analytical path of the referred system. Valve V2 is switchedon by timer T2, similarly to V1. However, the time interval which V2 remains on, must be longer than V2, to ensure proper sample exchange and cleaning of the analytical path. Upon shutting down V1 and keeping V2 on for a longer time, the carrier solution will continue flowing through c2, discarding all the residue generated during the exchange of the sample solution. All optimization conditions of the analyzes were defined experimentally.

Use of the IF system to verify the presence of microorganisms in the sample solutions:

Blank solution - 10 mL in minimal medium + 10 mL of peptone water, used as a blank formicroorganisms;

Reference Solution - 10 mL in minimal medium + 9 mL of peptone water 1 mL of *Escherichia coli* bacteria suspension, which is used as a reference condition;

Sample solution AmA - 10 mL in minimal medium + 9 mL of peptone water and 1 mL of thesample AmA solution;

Sample solution AmB - 10 mL in minimal medium + 9 mL of peptone water and 1 mL of sample AmB solution.

After the inoculations, each solution was read to evaluate their initial condition. Then all were transferred to a thermostatic bath, being maintained throughout the analysis time at a temperature around 37°C, a condition suitable to proliferation of possible microorganisms in all solutions.

Isolation of microorganisms: The colonies with different characteristics were selected and inoculated in a petri plate with a nutrient agar using the technique of stretch striation, with the aid of an inoculation loop, then incubated in a bacteriological oven at a constant temperature of 37°C for 24 hours.

Identification of microorganisms: After isolation the bacteria were submitted to Gram staining to identify their morphological and structural characteristics. Afterwards the biochemical identification, was carried out with the aid of the inoculation loop and transferred to the petri plate with MacConkey, Lactose and Mannitol agar for the identification of *Escherichia coli, Salmonella Sp* and *Staphylococcus aureus*, followed by incubation in the oven at 37°C for 24 hours. Then the typical observations of each microorganism were made.

RESULTS AND DISCUSSIONS

According to the results presented in Figure 3, it was possible to prove that the IF, proposed with UV/Vis spectrophotometric detection, was efficient to detect the presence of microorganisms in the solutions of lettuce samples. The tests were based on monitoring the proliferation of possible microorganisms, present in the samples under analysis.

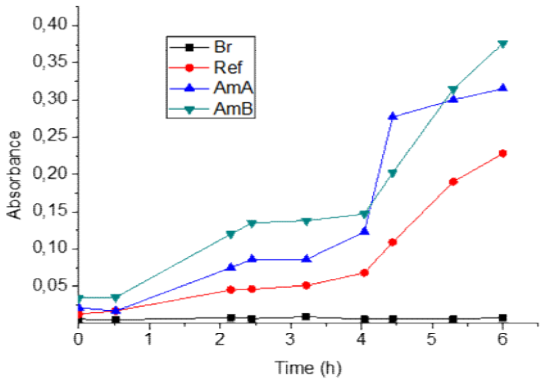

Figure 3. Evaluation for the detection and proliferation of microorganisms in the blank (Br) solution,reference (Ref) and lettuce samples (AmA) and (AmB).

The solutions of samples AmA and AmB presented similar results with significantly higher sensitivity than in the reference solution, thus suggesting the presence of more than one type of microorganisms with characteristics similar to those of Escherichia coli bacterium. As for the blank solution, the signal remained constant throughout the analysis time, thus demonstrating the culture medium used had not been contaminated with microorganisms. Therefore based on the proliferation of the microorganisms in the sample solutions, it was possible to prove the application of the IF system with spectrophotometric detection to detect the presence of microorganisms in lettuce sample solution. Since when the population of microorganisms increases, it generates an increase in turbidity of the medium and, consequently, a proportional increase in the absorption of electromagnetic radiation. Figure 4 shows the variation in turbidity, as a function of the proliferation of microorganisms in a sample solution. The test was performed by inoculating 1 mL of the AmA sample solution in 15 mL of the Minimum culture medium. This was kept in a water bath for 08 hours, being photographed right after inoculation, and in sequence every 4 hours. From left to right a gradient in the turbidity of the solution can be visualized, generated as a function of the population growth of the microorganisms over time.

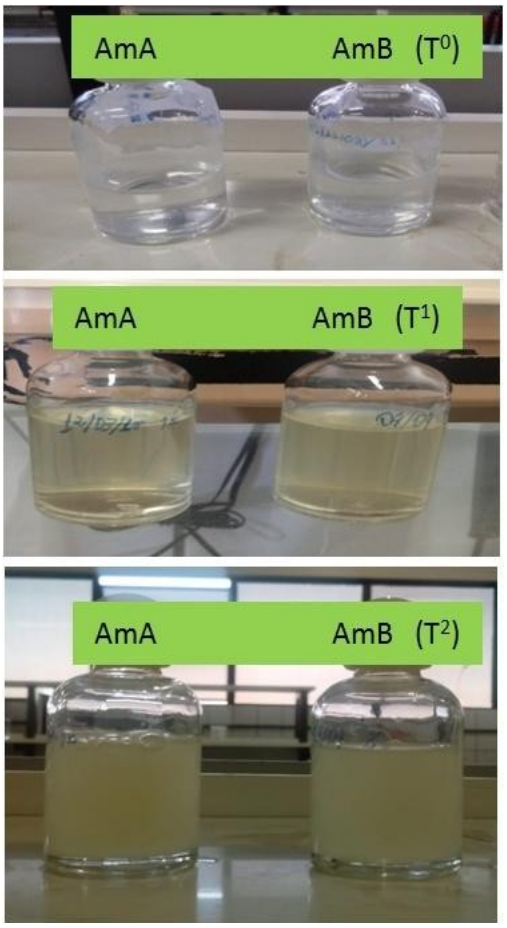

Figure 4. Visual evaluation of turbidity variation in sample solution (AmA) and (AmB) overtime. T^0, T^1 and T^2 = time 0,4 and 8 hours respectively, after the inoculation process

Microbiological analysis: After verifying the presence of microorganisms using the IF system, microbiological analyzes were carried out to identify them, the sample solutions were classified as described below: AmA sample referring to the first collection of lettuce sample and AmB sample, referring to the second collection. The Table I shows the numbers of colonies on the petri plates, where nutrient media inoculations were carried out, with the sample solutions in different dilutions. For dilution 10^0 and 10^{-1}, it was not possible to quantify the number of colonies, due to the high concentration, resulting in their overlap. According to the convention of the Food and Drug Administration, the North American agency that controls food and medicines, counting must be performed on plates containing 25 to 250 colonies, but microbiologists choose to count between 30 and 300 colonies, which are used in the current analysis. The results are within this value established in the 10^{-3} dilution, both in the AmA and AmB samples. In the 10^{-2} dilution, the number of colonies was above the established value, preventing its use. In the AmA sample, the colonies of isolated microorganisms had a rounded purple appearance, with a colony referring to a type of microorganism that developed on the nutrient agar. The AmB sample developed colonies related to two types of microorganisms, one with the same characteristics as the AmA sample, and the other with a yellowish round shape. The identification of microorganisms in the samples, was based on the development of colonies typical for the *Escherichia coli* bacteria, forming colonies in purple color, which showed pink color on MacConkey agar, and for the bacterium *Staphylococcus aureus*, which developed fermentation in a Mannitol agar changing the pH of the medium, leaving the colonies and the golden yellow medium (Figure 5).

Table 1. Number of colonies of microorganisms in AmA and AmB solutions, inoculated in petre platescontaining medium with nutrients

Samples	AmA				AmB			
Diluitions	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{0}	10^{-1}	10^{-2}	10^{-3}
Ufc/100 µL	countless	countless	357	221	countless	countless	328	261

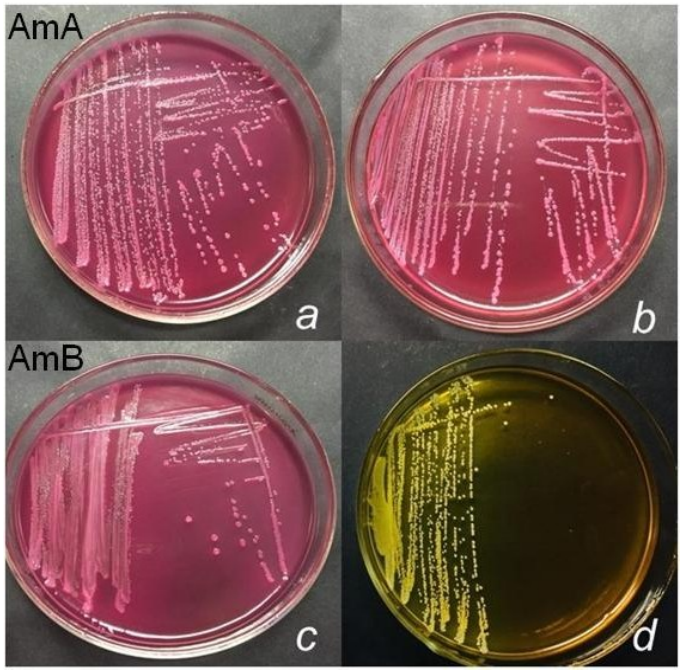

Figure 5. Development of the microorganisms Escherichia coli and Staphylococcus aureus in the solutionsof the AmA and AmB samples

In the Lactose culture medium, the propagation of microorganisms did not occur, thus ruling out the presence of *Salmonella sp*, since it does not ferment lactose and forms transparent colonies. Therefore, according to the results presented in Figure 4, in the AmA sample the presence of the *Escherichia coli* bacteria were found, on MacConkey agar (plates a and b) and in the AmB sample, the presence of the *Escherichia coli* bacteria was verified in MacConkey agar (plate c) and Staphylococcus aureus on Mannitol agar (plate d).

CONCLUSION

Microbiological analyzes enabled the identification of *Escherichia coli* and *Staphylococcus aureus* bacteria, which reveals the lack of hygienic-sanitary conditions, which can lead to contamination as well as the proliferation of microorganisms resulting in possible sources of foodborne diseases. The IF system showed positive results regarding the identification of the presence of microorganisms in the samples under analysis, monitoring their population development under given conditions, thus making it a new methodology for this type of analysis. Therefore, the results achieved in this work demonstrated that it is necessary to properly clean the lettuce for human consumption, in order to avoid possible damage to health due to contamination with microorganisms.

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