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RESISTANCE IN CHLORINATED WATER BY *Salmonella* sp. ISOLATED FROM VEGETABLES MINIMALLY PROCESSED

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ABSTRACT: The minimum processed greeneries must come to the consumer with the same properties of the product *in natura,* useful life extended and secure microbiologically. The objective was to evaluate the MPG microbiologically quality, and to submit the pathogenic microorganism to resistance tests in chlorine water. Was analyzed 25 samples of MPG mixed in Toledo-PR. The analysis done were the determination of fecal or total coliforms**,** mesophyll aerobic microorganisms, presence of *Salmonella* sp.*,* Sorology, tests of susceptibility and resistance tests in chlorine water. The results indicated that the 25 samples were contaminated by fecal or total coliforms**,** 24 of these presented total coliforms and 18 fecal coliforms higher or the same than 100 NMP/g and at the total counting of mesophyll aerobic bacterium, the 25 samples were contaminated, presenting a range of 14×10^5 UFC/g. Was observed the presence of *Salmonella* in 6 samples and these were sensitive to the antibiotics tested. After this, were submitted to the resistance tests in chlorine water, where was possible to observe its resistance at the concentrations 100, 125 e 150 ppm. Thus, 72% of MPG analyzed, were unavailable to human consumption, due to the presence of microorganisms potentially pathogenic. What can be suggested is the necessity of the application of a control over the poison used and about the temperature, to assure a healthy product to the costumer.

Key words: minimum processed greeneries, Salmonella, resistance.

RESUMO: "Resistência em água clorada por *Salmonella* sp. isolada de hortaliças minimamente processadas". As hortaliças minimamente processadas (HMP) devem chegar ao consumidor com as mesmas propriedades do produto *in natura*, vida útil prolongada e seguras microbiologicamente. O objetivo foi avaliar a qualidade microbiológica de HMP e submeter os microrganismos patogênicos a testes de resistência em água clorada. Analisou-se 25 amostras de HMP mistas em Toledo/PR. A determinação de coliformes totais e fecais, microrganismos aeróbios mesófilos, presença de *Salmonella* sp., sorologia, antibiograma e testes de resistência em água clorada foram analisadas. Os resultados indicaram que as 25 amostras estavam contaminadas por coliformes totais e fecais, destas 24 apresentavam coliformes totais e 18 coliformes fecais superiores a 100 NMP/g e na contagem total de bactérias aeróbias mesófilas as 25 amostras estavam contaminadas, apresentando uma média de 14×10^5 UFC/g. Foi observada a presença de *Salmonella* em 6 amostras e estas mostraram-se sensíveis aos antibióticos testados. Após foram submetidas aos testes de resistência em água clorada, podendo observar sua resistência nas concentrações de 100, 125 e 150 ppm. Assim, 72% das HMP analisadas estavam impróprias para o

consumo humano, por apresentarem microrganismos potencialmente patogênicos. Há necessidade da aplicação de um controle sobre o sanitizante utilizado e sobre a temperatura, assegurando um produto saudável ao consumidor.

Palavras-chave: hortaliças minimamente processadas, *Salmonella*, resistência.

1.0 - INTRODUCTION

The consumption of vegetables minimally processed is increasing in the last years, in function of the consumers' interest for more natural foods, nutritious and ready for the consumption, that due to the new tendency of the modern life (Vitti et al., 2004).

It is considered a vegetable minimally processed that prepared according to units of simple operation, that maintain the same physical properties, chemistries, nutritional and sensorial of the product *in natura*, lingering useful life, besides safety in relation to the transmission of diseases (Oliveira et al., 2005).

Fantuzzi et al., (2004) it puts that the time of useful life differs among the types of products processed minimally, but it varies among 7 to 20 days, when maintained in the recommended temperatures.

Microbiological quality of the foods minimally processed is related to his/her safety, not presenting contamination for pathogenic agents or with toxicological profile (Pinheiro et al., 2005).

The presence of injured cells and the loss of cellular components during processing operations, trickening provide optimal conditions for the development of micro-organisms (Smanioto et al., 2009).

According resolution RDC no. 12, of January 02, 2001, of the Ministry of Health, and of the National Agency of Sanitary Surveillance (ANVISA), it establishes that vegetables, in natura, prepared, sanitized, refrigerated or frozen for direct consumption, they should present a more probable number (NMP) of coliforms in to 100/g and the absence of Salmonella in 25g (Brasil, 2001).

Minimally processed marketed they are sanitized in solution of chlorine (120 to 150 ppm), packed and refrigerated (5 to 10°C) and disposed for the consumption (Rosa et al., 2004).

Many parts of the world the fruits and vegetables often have been incriminated in foodborne illness, this potential to be sources of pathogenic microorganisms. The leafy vegetables, especially lettuce, have been identified as carriers of pathogens such as *Escherichia coli, Listeria monocytogenes,* Salmonella and Shigella (Santos; Junqueira; Pereira, 2010).

In Brazil, from 1998 to 2000, 192 outbreaks of alimentary infection were registered with 12.188 sick and 3 deaths, having been the *Salmonella* sp. the responsible for most, with incidence in 76,56% of these occurrences. The vegetables were responsible for 19 (9,9%) outbreaks (Sirveta, 2007).

As Germano; Germano (2003) "In the etiological agents' of pathologies enteric health public, big part they are transmitted through vegetables, vegetables and polluted fruits, standing out among them helmints, protozoa, bacteria mushrooms and virus."

Pathologies transmitted by foods can be acted and divided in toxinose, infection and toxinfecction. In the toxinose it happens the ingestion of toxins bacterial pre - formed in the foods, due to the multiplication of bacteria toxinogênicas, like the *Staphylococcus aureus*, *Clostridium botulinum* and *Bacillus cereus* emetic. In the infection there is a clinical picture due to the ingestion of microorganism pathogenic that you/they multiply in the treatment gastrointestinal, producing toxins or aggression to the epithelium, as *Salmonella* sp., *Shigella* sp., *Escherichia coli*, and pathogenic vibrios, among others. Already in the toxinfection it happens the ingestion of increased amounts of bacteria in the vegetative form that liberate toxins in the treatment gastrointestinal to sporulate, however without colonizing, like the *Clostridium perfringens* and classic *Bacillus cereus* (Silva, 2005).

The presence of minimally processed vegetables increases the oxidation and the possibility of contamination by microorganisms, existing the prevention need and control during whole the handling, including the production process and packaging of the products (Lima et al., 2003).

Microbial growth can also be controlled with the use of vegetables of great quality,

sanitization and low temperatures (Aguila et al., 2006).

In Brazil, studies have been verified green vegetables with high degree of contamination for fecal coliforms that they can be coming of flaws in the productive process, transport, storage and manipulation of the same ones (Guimarães et al., 2003).

Contrarily most of the techniques of processing foods that stabilize the life of shelf of the products, the minimum processing of vegetables increases his perishability, taking to a faster deterioration in case it is not conditioned in a correct way (Bonnas et al., 2005).

Furlaneto et al., (2005) puts that 20% of the vegetables destined for the human consumption suffer microbiological alterations, and that during his commercialization they can be evidenced more than 250 types of microorganisms, between the deteriorating and the potentially pathogenic.

A quality control, observing the Good Practices of Production (GPP) for subsequent application of a preventive system, about the Analysis of Dangers and Critical Points of Control (DCPC) it is essential to assure a healthy product with high quality and useful life (Damasceno; Stamford; Alves, 2001).

The possibility to control the dangers and to maintain them in acceptable levels without risk for the consumer's health depends on the commitment of who produces and of the effectiveness of the authorities that legislates and they supervise (Freitas et al., 2004).

Therefore, it can be affirmed that the control hygienic-sanitarium of the foods allows to prevent the illnesses that can reach the man through his consumption, besides to assure his quality and to reduce the waste of the same ones (Germano; Germano, 2003).

Due to the increase of cases of infections provoked by polluted vegetables, the present work aimed at to verify the occurrence of Salmonella sp. in vegetables minimally processed and his resistance profile in chlorinated water.

2.0 - MATERIALS AND METHODS

25 samples of vegetables mixed minimally processed were collected, in the supermarkets of Toledo - PR, in the period of March to August of 2011. After each collection, the samples were transported to the laboratory of microbiological analysis of the Universidade Paranaense - UNIPAR, in isothermal boxes so that the temperature of the product stays her constant. Before the beginning of each analysis it happened the sanitization of the packing with alcohol 70%, open in asepsis conditions (flow to laminate) (Silva; Junqueira; Silveira, 2001).

2.1 - Total counting of aerobic mesophil bacteria - Taken a bracket from 25g of each sample to be analyzed, this diluted in 0,1% peptone water sterile and, later, made the dilutions 10^{-3},10^{-4} and 10^{-5} for the inoculation of the culture means used in the experiment. After the dilution of the samples in peptone water, they were pippeted brackets of 0,1 mL of the dilution 10-5 and inoculated in plates of Petri in copy, for total counting of mesophil bacteria, being increased to each plate about 15-20 mL of agar (PCA), previously melted and cold to 45°C, mixed in circular movements. After complete solidification, the plates were incubated to 37°C, for 48 hours. Elapsed that period there was the counting of the total number of present colonies in the plates, expressed in colony forming units per gram - CFU/g (Silva; Junqueira; Silveira, 2001).

2.2 - Determination of total and fecal coliforms - The most probable number (NMP) of total coliforms it was certain through the technique of multiple tubes being used the broth lauril sulfate triptose initially (LST). The cultures in broth LST with production of gas and growth were transferred for tubes of brilliant green broth (VB). The determination of NMP of total coliforms was accomplished starting from the number of tubes with production of gas and growth in VB, being used the table of NMP (Silva; Junqueira; Silveira, 2001). For the determination of the number NMP of fecal coliforms was inoculated the cultures with production of gas and growth in LST in broth Escherichia coli (EC). Starting from the number of tubes with production of gas and growth in broth EC, was certain NMP of fecal coliforms (Silva; Junqueira; Silveira, 2001).

2.3 - Detection of *Escherichia coli* - Of each tube of EC with production of gas and growth, it was grooved in plates of ágar blue eosina of metilene (EMB), for characterization of typical colonies of Escherichia coli. Later it was accomplished the coloration of Gram and inoculation in the means for the biochemical proofs of indol, citrate, red methyl and Voges-Proskauer (Silva; Junqueira;

Silveira, 2001).

2.4 - Detection of *Salmonella* - After the retreat of the bracket for the determination of total and fecal coliforms, the samples in the primary dilution were destined for the detection Salmonella being incubated to 35ºC by 18 hours. After this period, brackets were transferred for broth tetrationato (TT) and broth selenito-cistina (SC). Of each tube, she preceded the isolation of typical colonies in ágar bismuth sulfito (BS), ágar xilose lisina desoxicolato (XLD) and ágar entérics of Hectoen (HE). Suspicious Colonies were grooved in sloping ágar iron trípice sugar (TSI) and lisina iron (he/she READ). The isolated ones that you/they presented characteristic reactions of Salmonella sp. they were submitted to the coloration of Gram and the biochemical identification through the following tests: urease, indol, red of methyl and Voges-Proskauer, citrate, fermentation of the dulcitol, of the sucrose and of the lactose and mobility (Silva; Junqueira; Silveira, 2001).

2.5 - Automated confirmation, antibiogram and sorology - The isolated ones that you/they presented characteristic results for *Salmonella* sp. they were confirmed by the Micro Scan Dried Enterobacteriacea MIC/Combo System (Dade Behring Inc., Deerfield, IL). The antibiogram was obtained by the microdilution method (Solemnity Scan 4), being tested the sensibility by the antibiotics: Ampicilina, Ceftriaxona, Ciprofloxacina, Gentamicina, Imipenem, Levofloxacina and Trimetropim/Sulfametoxazol. The classification is represented for resistant, middleman and sensitive to the test antimicrobiano. The sorology for classification of the serotypes of *Salmonella* sp. it was accomplished by the agglutination method in serums (Probac, 2005).

2.6 - Resistance profile in chlorinated water - For the resistance analysis in chlorinated water, a new culture "overnight" was accomplished starting from the isolated samples and stored in glicerol. The reactivation happened in 5 mL of Nutritious Broth, each sample being washed three times with solution of Salt bed 0,9%, then ressuspended in salt bed 0,9% the concentration of 1×10^{7} UFC/mL, standardized through the method of visual comparison with turbidity pattern corresponding to 0,5 of the scale of Mac Farland. Prepared the hypochlorite solutions in the concentrations: 100/125/150/175 and 200 ppm, there was the homogenization of 1 mL of the suspension of microorganisms with 1 mL of the hipoclorito concentrations, for 1 minute, after stop the reaction with 0,5 mL of tiossulfato of sodium 0,7 mg/mL. Then it was plaqueado 10 µl of each sample in nutritious ágar, incubated to 37°C by 24:00. After that period the counting of the number of colonies was accomplished (Scoaris et al., 2008).

3.0 - RESULTS AND DISCUSSION

The 25 analyzed samples were polluted for total and fecal coliforms (Table 1), of these 24 (96%) they presented total coliforms and 18 (72%) superior fecal coliforms to 100 NMP/g. That index is used to evaluate the hygienic conditions of the food, and high countings mean contamination powder-processing, besides cleaning and deficient sanitization of equipments, resulting in great risk of the pathogen presence potentially dangerous as *Salmonella* sp. (Oliveira et al., 2005).

Smanioto et al., (2009) cites in their work that seven samples had toatal coliform counts equal to or greater than 1.100 NMP.g^{1} indicating lack of compliance with Good Manufacturing Practices

It was verified in the total counting of aerobic mesophile bacteria, that the 25 samples were polluted (Table 1), presenting an average of 1,4×106 UFC/g. For minimally processed vegetables, legislation doesn't exist determining limits of allowed countings, but it can be affirmed that amounts above 5×102 healthy UFC/g completely undesirable, in reason of the risk of presence of pathogenic and deteriorating microorganisms and/or (Vitti et al., 2004).

In agreement with Oliveira et al., (2005), the high counting of mesophil aerobic bacteria in perishable foods can indicate excessive contamination of the matters - cousins; sanitization badly accomplished; inadequate hygiene in the production; and use of inappropriate time/temperature during the production or conservation of the products. Like this, contributing to the biological risk once a lot of bacteria pathogenic transmitted by foods are mesophils. The presence of *Escherichia coli* was not detected in none of the analyzed samples.

The presence of *Salmonella* was observed in 24% of the samples (Table 1), indicating that the marketed product presents great risk potential to

the consumer's health. According to Furlaneto et al. (2005), those bacteria are the main agents involved in outbreaks of alimentary origin, and they mention the vegetables as source common of this patógeno. The presence of pathogens in minimally processed vegetables is preoccupying, tends in view that they are consumed without thermal treatment, and the packings of these products bring the "Ready message for the consumption."

Table 1- Results of the microbiological analysis of minimally processed vegetables.

Sample	Total Coliforms (NMP/g)	Fecal Coliforms (NMP/g)	*Salmonella* sp.	Aerobic Mesophils (CFU/g)
1	240	240	Absence	$1,0\times10^6$
2	=2400	=2400	Absence	$1,1\times10^6$
3	240	240	Absence	$0,6\times10^6$
4	240	240	Absence	$0,9\times10^6$
5	=2400	93	Absence	$0,8\times10^6$
6	240	240	Absence	$1,6\times10^6$
7	240	240	Absence	$2,9\times10^6$
8	=2400	150	Absence	$1,4\times10^6$
9	93	<3	Absence	$0,3\times10^6$
10	=2400	=2400	Presence	$2,5\times10^6$
11	460	240	Absence	$1,5\times10^6$
12	210	240	Absence	$2,2\times10^6$
13	=2400	=2400	Presence	$2,3\times10^6$
14	460	150	Absence	$1,2\times10^6$
15	=2400	23	Absence	$0,9\times10^6$
16	=2400	240	Absence	$1,1\times10^6$
17	150	23	Absence	$0,6\times10^6$
18	460	<3	Absence	$0,3\times10^6$
19	240	<3	Absence	$0,5\times10^6$
20	=2400	240	Absence	$1,2\times10^6$
21	=2400	=2400	Presence	$2,2\times10^6$
22	=2400	=2400	Presence	$2,4\times10^6$
23	=2400	=2400	Presence	$1,7\times10^6$
24	460	<3	Absence	$0,8\times10^6$
25	=2400	=2400	Presence	$2,0\times10^6$

NMP/g – most probable number per gram;CFU/g – colony forming units per gram

The percentile of irregularities observed in the present study didn't differ substantially of the described in the national literature. Pinheiro et al. (2005), in their analyses of minimally processed fruits they obtained contamination for Salmonella sp. in 25% of their samples, and in 28% fecal coliforms with superior values to 5×10^2 NMP/g. Oliveira et al., (2005), when evaluating the hygienic-sanitary quality of minimally processed lettuces, they verified that 73% of the samples presented high countings of fecal coliformes (>103 NMP/g), as well as for aerobic mesophils (>106 UFC/g), and presence of *Salmonella* sp. in 93% of the samples.

The isolated ones were identified as Salmonella sp. and they were shown sensitive the all the tested antibiotics (Table 2). After they were submitted to the resistance tests in chlorinated water (Table 3), where she can observe his/her resistance in the concentrations 100, 125 and 150 ppm.

The Ministry of the health, Brazil (2001) and of the National Agency of Sanitary Surveillance (ANVISA) it extols the use of concentrations between 100 and 200 ppm for higienização of vegetables, it happens that usually used him/it in the industry of minimally processed vegetables is between 120 and 150 ppm, what possibly explains the presence of Salmonellas sp. in 24% of the analyzed samples.

The implantation of an effective system of control, through a program of Analysis of

Dangers and Critical Points of Control (ADCPC) it is fundamental for the knowledge and prevention of the contamination and of the microbial growth in minimally processed vegetables (Bonnas et al., 2005).

The Good Practices of Production (GPP), as the wash in water chlorinated controlled an automatic one and well projected cut machine, training of the operators, among other, they can contribute to the reduction of the initial and subsequent microbiological load. Just a strategic combination of those procedures can drive the safety's effective improvement and quality of minimally processed vegetables (Bonnas et al., 2005).

Table 2 - Minimal inhibitory concentration of *Salmonella* sp. isolated from minimally processed vegetables.

Antibiotic	Susceptibility (µg/mL)			Isolates with the indication of MIC					
				Minimally Processed Vegetables					
				Salmonella sp.					
	S	I	R	A1	A2	A3	A4	A5	A6
Amikacin	≤ 16	16-32	≥ 32	≤ 8	≤ 8	≤ 8	=8	≤ 8	≤ 8
Ampicilina	≤ 8	8-32	≥ 32	≤ 2	= 2	≤ 2	> 2	≤ 2	≤ 2
Amp/Sulbactam	≤ 8/4	8/4-32/16	≥ 32/16	≤ 8/4	≤ 8/4	≤ 8/4	≤ 8/4	≤ 8/4	≤ 8/4
Aztreonan	≤ 8	8-32	≥ 32	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8
Cefalozina	≤ 8	8-32	≥ 32	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8	= 8
Cefepima	≤ 8	8-32	≥ 32	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8
Cefotaxima	≤ 8	8-64	≥ 64	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4
Ceftazidima	≤ 8	8-32	≥ 32	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2
Ceftriaxona	≤ 8	8-64	≥ 64	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4
Cefuroxima	≤ 4	8-32	≥ 32	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4
Ciprofloxacina	≤ 1	1-4	≥ 4	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Gentamicina	≤ 4	4-8	≥ 8	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Imipenem	≤ 4	4-16	≥ 16	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4
Levofloxacina	≤ 2	2-8	≥ 8	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2
Meropenem	≤ 4	4-16	≥ 16	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4
Moxifloxacina	≤ 2	2-		≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2
Piperacilina/Tazobactan	≤ 16/4	16/4-128/4	≥ 128/4	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8
Piperacilina	≤ 16	16-128	≥ 128	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16
Ticarcilina/ Ác. Clavulânico	≤ 16/2	16/2-128/2	≥ 128/2	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16	≤ 16
Tobramicina	≤ 4	4-8	≥ 8	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2
Trimetropim/Sulfametoxazo	≤2/38	2/32-8/152	≥ 8/152	≤ 0.5/9.5	≤ 0.5/9.5	≤ 0.5/9.5	≤ 0.5/9.5	≤ 0.5/9.5	≤ 0.5/9.5

S= Susceptible; I= Intermediary; R= Resistant
MIC – minimal inhibitory concentration; µg/mL – micrograms per mL; Amp – ampicilin; Ác. - ácido

Table 3 - Results of the resistance tests in chlorinated water of *Salmonella* sp. Isolated from minimally processed vegetables.

Sample	Microbial growth in the hypochlorite concentrations				
	100 ppm	125 ppm	150 ppm	175 ppm	200 ppm
1	300 UFC/g	11 UFC/g	03 UFC/g	Absentee	Absentee
2	21 UFC/g	8 UFC/g	01 UFC/g	Absentee	Absentee
3	10 UFC/g	5 UFC/g	02 UFC/g	Absentee	Absentee
4	11 UFC/g	5 UFC/g	02 UFC/g	Absentee	Absentee
5	14 UFC/g	7 UFC/g	01 UFC/g	Absentee	Absentee
6	48 UFC/g	9 UFC/g	02 UFC/g	Absentee	Absentee

ppm – parts per million; CFU/g – colony forming units per gram

4.0 - CONCLUSION

Of the minimally processed vegetables analyzed, 72% were inappropriate for the human consumption, because they present potentially pathogenic microorganisms, suggesting the need of the application of a control about the concentration of the used sanitizing and on the temperature (≤ 5°C), to assure a healthy product to the consumer.

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