

Assessment of Microbiological Contamination of Fresh, Minimally Processed, and Ready-to-Eat Lettuces (*Lactuca sativa*), Rio de Janeiro State, Brazil

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Abstract: This study aimed to assess the microbiological contamination of lettuces commercialized in Rio de Janeiro, Brazil, in order to investigate detection of norovirus genogroup II (NoV GII), *Salmonella* spp., total and fecal coliforms, such as *Escherichia coli*. For NoV detection samples were processed using the adsorption–elution concentration method associated to real-time quantitative polymerase chain reaction (qPCR). A total of 90 samples of lettuce including 30 whole fresh lettuces, 30 minimally processed (MP) lettuces, and 30 raw ready-to-eat (RTE) lettuce salads were randomly collected from different supermarkets (fresh and MP lettuce samples), food services, and self-service restaurants (RTE lettuce salads), all located in Rio de Janeiro, Brazil, from October 2010 to December 2011. NoV GII was not detected and PP7 bacteriophage used as internal control process (ICP) was recovered in 40.0%, 86.7%, and 76.7% of those samples, respectively. *Salmonella* spp. was not detected although fecal contamination has been observed by fecal coliform concentrations higher than 10² most probable number/g. *E. coli* was detected in 70.0%, 6.7%, and 30.0% of fresh, MP, and RTE samples, respectively. This study highlights the need to improve hygiene procedures at all stages of vegetable production and to show PP7 bacteriophage as an ICP for recovering RNA viruses' methods from MP and RTE lettuce samples, encouraging the evaluation of new protocols that facilitate the establishment of methodologies for NoV detection in a greater number of food microbiology laboratories.

Keywords: bacterial contamination, internal control, lettuce, norovirus, RT-PCR

Practical Application: The PP7 bacteriophage can be used as an internal control process in methods for recovering RNA viruses from minimally processed and ready-to-eat lettuce samples.

Introduction

Leafy green vegetables are important components of a healthy diet, providing important vitamins, minerals, and phyto-nutrients (Mercanoglu and Halkman 2011). Because of these potential benefits, governments around the world have been promoting the consumption of fresh vegetables and their minimally processed (MP) products to prevent diseases (FAO and WHO 2008a). However, there has been an increasing recognition of foodborne disease outbreaks linked to the consumption of ready-to-eat (RTE) vegetables (FAO and WHO 2008b; Berger and others 2010). Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) convened an Expert Meeting to discuss how to address adequately the scientific advice on microbiological hazards associated with fresh produce. Leafy green vegetables were identified as the commodity group of highest concern from a microbiological safety perspective. Norovirus (NoV), *Escherichia coli* O157:H7, and *Salmonella* spp. were included among

the more common pathogenic microorganisms that can be transmitted to humans by consumption of these products (FAO and WHO 2008a).

Leafy green vegetables are produced in diverse and complex ways, and can be exposed to microbial contamination at various stages during the production, as preharvest, harvest, packing, processing, storage, or during distribution. Preharvest contamination can occur directly or indirectly via animals, water, soil, dirty equipment, and human manipulating. However, the fecal contamination of the irrigation water and the use of manure or compost fertilizer to fields are the most important vials of contamination (FAO and WHO 2008a; Oliveira and others 2010). In Brazil, from 2000 to 2011; 7234 foodborne outbreaks were reported, of which the leafy green vegetables have been implicated as a vehicle of contamination in 96 (1.33%). The most common etiologic agent isolated in the foodborne outbreaks is *Salmonella* spp. that were identified in 1660 (22.95%) outbreaks. *E. coli* appears in the 4th position, being responsible for 411 outbreaks (5.68%) (Brasil 2013).

The NoV genus belongs to the *Caliciviridae* family and is divided into 5 genogroups (G), which GI, II, and IV are known to infect humans (Zheng and others 2006). NoV is one of the major causes of acute gastroenteritis worldwide and is responsible for up to 1.1 million hospitalizations with an estimated mortality rate of approximately 218,000 deaths per year (Patel and others 2008).

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NoV GII has been the most prevalent in cases of foodborne infections (Patel and others 2009). In Brazil, GII.4 strain is the most prevalent NoV genotype circulating and responsible for the major of NoV foodborne outbreaks (Ferreira and others 2008; Barreira and others 2010; Fioretti and others 2011).

Many well-documented foodborne outbreaks due to NoV have been examined in epidemiological investigations (FAO and WHO 2008b). However, the direct detection of NoV in the implicated foods is generally unsuccessful (Scherer and others 2010). To improve microbiological monitoring of food quality and to assess the true role of food in viral transmission, standardized methods need to be developed for use in reference laboratories and for monitoring foodstuffs (Crocini and others 2008). New approaches have focused on virus extraction, concentration and detection by using polymerase chain reaction (PCR) technology in order to improve sensitivity (Morales-Rayas and others 2010; Sánchez and others 2012).

The current study aims to assess NoV GII and bacteriological contamination by *Salmonella* spp., total and fecal coliform (and the *E. coli* identification) levels in samples of fresh, MP, and RTE lettuces commercialized in the State of Rio de Janeiro, Brazil. The bacteriophage PP7 was also evaluated as internal control process (ICP) of viral concentration methodology, since it is regarded as a suitable surrogate for human enteric viruses from water samples (Rajal and others 2007a, 2007b).

Materials and Methods

Viruses

NoV (Hawaii virus) GII.1 strain prototype and PP7 bacteriophage (ATCC 15692-B2) were used for constructing the standard curve (SC) of the quantitative assays. A 10% (w/v) positive fecal suspension containing NoV GII from the Regional Reference Gastroenteritis Laboratory collection, Oswaldo Cruz Institute, Rio de Janeiro-RJ, Brazil, and the PP7 bacteriophage were used for spiking experiments. Titers of both viruses were established by real time PCR based on SC, represented by the absolute number of genome copies (gc)/ μL (Yin and others 2001).

Samples of lettuce

A total of 90 samples of lettuce including 30 whole fresh lettuces, 30 MP lettuces, and 30 raw RTE lettuce salads were randomly collected from different supermarkets (fresh and MP lettuce samples), food services, and self-service restaurants (RTE lettuce salads), all located in Rio de Janeiro, Brazil, from October 2010 to December 2011. The MP are defined as lettuce samples previously washed and soaked with appropriated sanitizing chemical and sold in disposable plastic food containers, being those procedures performed by producers. Products were transported individually under refrigeration (4 to 8 °C) to the laboratory.

Viral concentration method

An adsorption-elution concentration method using negatively charged membranes was performed according to the protocol described by Fumian and others (2009) with one modification: the centrifugation step was removed and the samples were weighed (25 g) in sterile Whirl-Pak[®] Stomacher filter bags (Nasco, Fort Atkinson, Wis., U.S.A.) and homogenized in a Stomacher[®] apparatus (Seward, Fisher Scientific, Sainte-Foy, Quebec, Canada) as described by other authors (Scherer and others 2010; Stals and others 2011; Sánchez and others 2012). The rinse fluid left in the filter compartment of the bag was used to perform the analysis.

Each commercialized lettuce product was inoculated with 50 μL of PP7 bacteriophage viral suspension ($\approx 3 \times 10^5$ gc) as an ICP.

RNA extraction, cDNA synthesis, virus detection, and quantification methods

Viral RNA was extracted from 140 μL of the 2 mL final eluate using QIAamp[®] viral RNA mini kit (Qiagen[®], Valencia, Calif., U.S.A.) in accordance with the manufacturer's instructions to obtain a final volume of 60 μL . The High-Capacity kit (Applied Biosystems, Calif., U.S.A.) was used for cDNA synthesis. In each reaction, 12.5 μL of viral RNA extract was added to a 12.5 μL of RT reaction mixture containing: 1 \times buffer, 8 mmol/L of each dNTP, 62.5 U of MultiScribe[™] reverse transcriptase, and 2 \times random primers. The reverse transcription conditions were performed as such: 10 min at 25 °C, 2 h at 37 °C, and 5 min at 85 °C. For each reaction set-up, negative (DNA/RNA free water—BioBasic, Ontario, Canada) and positive (NoV GII or PP7 bacteriophage) controls were included. With the intention to investigate the presence of inhibitors in samples, cDNA was also prepared using a 1:10 RNA dilution.

Nov GII and PP7 bacteriophage detection was conducted using a TaqMan[®] technology of real-time quantitative polymerase chain reaction (qPCR) according to protocols described previously (Kageyama and others 2003; Fumian and others 2010). Primers and probes are shown in Table 1. Reactions were performed in duplicate using the ABI 7500 real-time PCR system (Applied Biosystems[®]) according to the manufacturer's instructions. The generation of plasmids and the construction of the SC were performed as described previously (Fumian and others 2009, 2010). The SC was created using tenfold serial dilutions of pCR[®]2.1-TOPO[®] vectors (Invitrogen, Van Allen Way Carlsbad, Calif., U.S.A.) containing either the ORF1/ORF2 overlap region of the NoV genome (5.0×10^6 to 5.0×10^0) or the PP7 replicase gene (1.0×10^7 to 1.0×10^1). Seminested PCR was also performed to detect NoV GII with primers JV13I, JV12Y, and NoroII-R (inner primer is specific for GII genotypes) which target the viral RNA-dependent RNA polymerase gene (Boxman and others 2006). All procedures comprised negative (DNA/RNA free water) and positive (NoV GII and PP7 bacteriophage) controls to avoid false results.

NoV GII and PP7 bacteriophage efficiency recovery

Recovery efficiency of both NoV GII and PP7 bacteriophage was evaluated by experimental assays of artificial contamination. Briefly, 3 aliquots of 25 g of whole fresh lettuce samples were seeded by direct application of 200 μL of NoV GII fecal suspension and PP7 bacteriophage particles suspension onto food surface. After 30 min in a laminar flow hood, in order to facilitate viruses attachment, samples were analyzed according to the methodologies above described. Unspiked samples were processed as negative controls. The titers of NoV GII in the fecal suspension and PP7 bacteriophage particle suspensions used for experiments were established by real time PCR based on SC that are represented by the absolute number of gc/ μL . Part of the NoV GII fecal suspension and PP7 bacteriophage suspension were applied to RNA extraction and quantification together with the contaminated aliquots. For each type of virus and negative controls, 3 independently experiments were carried out using phosphate saline buffer (PBS) as elution solution.

Recovery of NoV GII and PP7 bacteriophage were both quantitatively and qualitatively analyzed as described by Stals and others (2011). Quantitative analysis ("recovery efficiency") was

Table 1—Primers and probes used for real-time quantitative PCRs and nested PCR performed in this study.

Molecular method	Virus	Primer/Probe	Sequence (5'→3')	Location
Real-time quantitative PCR	NoV GII	COG2F	CARGARBCNATGTTYAGRTGGATGAG	5003 ^a
		COG2R	TCGACGCCATCTTCATTCACA	5100
	PP7	RING2-TP	FAM-TGGGAGGGCGATCGCAATCT-TAMRA	5048
		247 f	GTTATGAACCAATGTGGCCGTAT	247 ^b
		320 r	CGGGATGCCTCTGAAAAAAG	320
Nested PCR	NoV GII	274 probe	FAM-TCGGTGGTCAACGAGGAAC-TAMRA	274
		JV13I	TCATCATCACCATAGAAIGAG	4585 ^c
		JV12Y	ATACCACTATGATGCAGAYTA	4279
		NoroII-R	AGCCAGTGGGCGATGGAATTC	4495

Degenerate primers and probes are as follows: Y, C, or T; R, A, or G; B, not A; N, any. W, A, or T; K, G, or T; S, G, or C.

^aCorresponding nucleotide position in PP7 bacteriophage (accession nr. NC_001628).

^bCorresponding nucleotide position in human NoV (accession nr. AF145896).

^cCorresponding nucleotide position in human NoV (accession nr. M87661).

Table 2—Evaluation of recovery efficiency of PP7 bacteriophage and NoV GII from artificially contaminated whole fresh lettuce samples.

Viruses	Nr. of inoculated copies (mean ± SD) ^a (×10 ⁶)	Recovery copies (mean ± SD) (×10 ⁶)	Recovery efficiency ^b (mean ± SD) (recovery success rate) ^c
PP7	2934.29 ± 1861.44	21.36 ± 25.98	0.63 ± 0.43 (9/9)
NoV GII	16.69 ± 3.35	0.036 ± 0.029	0.24 ± 0.20 (6/9)

^aStandard deviation; ^b(# genomic copies recovered × 100)/# genomic copies inoculated in 25 g of lettuce sample; ^c # Positive real-time PCR reactions/ # performed real-time PCR reactions.

calculated by comparing the mean of NoV GII or PP7 bacteriophage gc recovered with the mean of gc inoculated. Qualitative analysis (“recovery success rate”) of NoV GII or PP7 bacteriophage recovery was performed by comparing the number of positive real-time PCR signals with the total number of reactions.

Bacteriological analysis

Microbiological assays using culture methods were performed according to standard methodologies described by the Food and Drug Administration’s Bacteriological Analytical Manual online. Counts of total and fecal coliforms were carried out by a multiple-tube fermentation technique (Feng and others 2002) and *Salmonella* spp. detection was performed as described by Andrews and Hammack (2011).

Statistical analyses

Prevalence, mean values, and standard deviations (SD) of all variables were calculated. A nonparametric Kruskal-Wallis test (KW test) followed by Dunn’s method of comparison was applied to verify the difference among the values of total and fecal coliforms among the fresh, MP, and RTE lettuce samples. The latter was also used to compare if recovery success rate of PP7 bacteriophage was different amid samples. Binomial test (2 proportions) was used to determine the proportion of *E. coli* in distinct types of samples. Differences were considered statistically significant when *P*-values were lower than 0.05. All statistical calculations were performed using Biostat 5.0 (Universidade Federal do Pará, Brazil, 2007).

Results

To evaluate the recovery of NoV GII and PP7 bacteriophage using the adsorption-elution concentration method with negatively charged membranes, identical sized lettuce samples were artificially spiked with both viruses. According to Table 2, the PP7

bacteriophage was detected in all contaminated samples showing a recovery efficiency that ranged from 0.12% to 2.08% with a mean of 0.63% ± 0.43%. NoV GII was detected in 6 out of 9 contaminated samples (66.7%) showing a recovery efficiency that ranged from 0.06% to 0.67%, with an average of 0.24% ± 0.20% (Table 2). The viruses were not detected in negative controls.

The investigation of microbiological contamination in 90 lettuce samples obtained in Rio de Janeiro did not reveal the presence of NoV GII by any of the methodologies (qPCR and nested PCR). Concerning the use of PP7 as ICP, it was observed a recovery of 67.8% with a significant difference (KW test; *P* = 0.011) according to the type of lettuce tested (Table 3). By using Dunn’s comparison method, no significant difference was observed between RTE salads and the others (*P* > 0.05). On the other hand, when comparing whole fresh and MP lettuce samples, a significant difference was noticed (*P* < 0.05), indicating that the method showed a lower performance for ICP recovery when applied to whole fresh lettuce samples.

Salmonella spp. was not detected in any of the 90 commercialized lettuce samples analyzed. Enumeration of total and fecal coliforms in lettuce samples were summarized in Table 4. Mean values of total coliforms in whole fresh samples were significantly higher (*P* < 0.05), while MP and RTE samples did not show a significant difference (*P* > 0.05). Mean fecal coliforms counts ranged from <3.0 to 1.5 × 10³, <3.0 to 1.5 × 10², and <3.0 to >1.1 × 10⁴ most probable number (MPN)/g in whole fresh, MP, and RTE lettuce samples, respectively. Difference among the average of fecal coliform count in MP samples was significantly lower than in whole fresh and RTE samples (*P* < 0.05). In turn, whole fresh and RTE lettuce samples did not present a significant difference (*P* > 0.05). *E. coli* was detected in 21 (70.0%), 2 (6.7%), and 9 samples (30.0%) of fresh, MP, and RTE lettuces, respectively. The prevalence of *E. coli* was significantly different according to the type of lettuce tested (*P* < 0.001).

Discussion

The advances on molecular methods for detection and quantification of NoV along with food security policies established in various countries from the last 3 decades resulted in a remarkable number of protocols for recovering those viruses from food (Crocini and others 2008; Fumian and others 2009; Morales-Rayas and others 2010; Scherer and others 2010; Sánchez and others 2012; Corrêa and Miagostovich 2013). The difficulty of recovering a small number of particles associated with the interference of different food matrices has been circumvented by the use of more sensitive detection methods such as real-time PCR and the use of

Table 3—PP7 bacteriophage recovery by qPCR of spiked commercialized lettuce product samples.

Type of lettuce	Nr. of inoculated copies (mean ± SD) ^a (×10 ²)	Recovery copies (mean ± SD) (×10 ²)	Recovery efficiency ^b (mean ± SD)	Minimum recovery efficiency	Maximum recovery efficiency	Recovery success rate ^c (%)
Fresh	1916.53 ± 756.14	31.51 ± 50.42	2.01 ± 3.29%	0.01%	10.82%	12/30 (40.0%)
MP ^d	1611.80 ± 728.08	9.97 ± 11.18	0.67 ± 0.88%	0.01%	4.36%	26/30 (86.7%)
RTE ^e salads	6320.35 ± 5822.97	16.80 ± 44.91	0.49 ± 0.69%	0.001%	2.39%	23/30 (76.7%)

^aStandard deviation; ^b(# genomic copies recovered from concentrate × 100)/# genomic copies inoculated in 25 g of lettuce sample; ^c# Positive real-time PCR reactions/ # performed real-time PCR reactions; ^dminimally processed; ^eready-to-eat.

Table 4—Total and fecal coliforms in lettuce samples.

Microorganisms	Type of sample	Microbial population ^a (% positive samples)					Nr. of positive samples (%) ^b
		<3.0	3.1 to 99	100 to 999	1000 to 11000	>11000	
Total coliforms	Fresh	0	0	0	15 (50.0)	15 (50.0)	30 (100.0)
	MP ^c	0	6 (20.0)	9 (30.0)	9 (30.0)	6 (20.0)	30 (100.0)
	RTE ^d salads	0	2 (6.7)	6 (20.0)	12 (40.0)	10 (33.3)	30 (100.0)
Fecal coliforms	Fresh	8 (26.7)	18 (60.0)	3 (10.0)	1 (3.3)	0	22 (73.3)
	MP	26 (86.7)	3 (10.0)	1 (3.3)	0	0	4 (13.3)
	RTE salads	8 (26.7)	16 (53.4)	4 (13.3)	1 (3.3)	1 (3.3)	22 (73.3)

^aMost probable number/g; ^b(# of positive samples/ # of samples) × 100; ^cminimally processed; ^dready-to-eat.

ICP allowing higher reliability of the results (Kageyama and others 2003; Mattison and others 2009; Stals and others 2011).

This study focused on PP7 bacteriophage as ICP rather than other viruses as Murine Norovirus 1 (MNV-1) (Stals and others 2011; Sánchez and others 2012; Corrêa and Miagostovich 2013), feline calicivirus (FCV) (Mattison and others 2009), MS2 phage (Scherer and others 2010), or Mengo virus (Uhrbrand and others 2010) since PP7 propagation is more accessible to food microbiology laboratories than cell cultures used to produce stocks of those viruses (Rajal and others 2007b). PP7 bacteriophage has also been successfully used in processes of concentrating viruses in aquatic matrices and have been chosen due to its similarity both by its size (25 nm) and its physicochemical properties to poliovirus, simulating the worst scenario for viral filtration (Rajal and others 2007b; Fumian and others 2010). Comparing with these others viruses used as IPC, Mattison and others (2010) detected the FCV in 83% of the RTE packaged leafy greens samples analyzed. This result was similar to our findings, since the PP7 bacteriophage was detected in 86.7% and 76.7% of MP and RTE lettuce samples, respectively. Scherer and others (2010) using MS2 phage as ICP reported a lower recovery rate, ranging from 6% to 10% for samples cut fruit and vegetables. On the other hand, Sánchez and others (2012) used the MNV-1 as ICP for in samples of fresh fresh-cut vegetables and detected the MNV-1 in all spiked samples.

The success of the initial experiments conducted to evaluate the PP7 recovery from the filtration methodology (100.0%) determined its use as ICP in the samples investigated. However, PP7 bacteriophage was not detected in all commercialized spiked lettuce samples, indicating the presence of inhibitors in the lettuce samples analyzed (Table 3). PP7 bacteriophage showed a higher success rate in MP and RTE lettuce samples compared to whole fresh lettuce samples, suggesting that the act of washing lettuces reduces the solids and the concentration of inhibitory substances onto matrix surfaces. Furthermore, PP7 bacteriophage adsorption to solids can occur, lowering the overall recovery (Rajal and others 2007a) as well as the clogging of filters due to a large quantity of debris that may be present in the samples, especially whole fresh ones, reducing the recovery rate (Victoria and others 2010). The effect of different food matrices on the viral quantification in food

products has been investigated by some authors and the results vary according to the type of food (Stals and others 2011). The nucleic acid dilution described as a common strategy used for environmental samples (Fumian and others 2010; Victoria and others 2010; Prado and others 2011) for overcoming inhibition of PCR amplifications was used in this study (data not shown) but it was not efficient enough to avoid the inhibitions observed, including recovering of NoV GII. One hypothesis raised to explain the low percentage of NoV GII recovery obtained is the presence of free RNA and defective viral particles in fecal suspension used to spike experiments. Those can be detected when subjected to nucleic acid extraction but are more susceptible to elimination during the concentration step. This leads to an underestimated calculation of the NoV GII recovery efficiency due to the heterogeneity of the fecal suspension (Sánchez and others 2012). Other methodologies vary greatly in the percentage of NoV recovery from different types of lettuce samples, ranging from 0.005% to 99% (Fumian and others 2009; Morales-Rayas and others 2010; Scherer and others 2010; Stals and others 2011; Sánchez and others 2012). The NoV detection and ICP results were similar to those reported by other researchers (De Giusti and others 2010; Scherer and others 2010) and stress the need to carry out further studies to establish the use of PP7 bacteriophage as ICP.

Concerning bacteriological results, the absence of *Salmonella* spp. in lettuce concurs with results of previous studies carried out with other types of Brazilian vegetables (Abreu and others 2010; Santos and others 2010), although *Salmonella* spp. is still the most common pathogens identified in outbreaks throughout the country (Brasil 2013). Studies reporting the presence of this pathogen in whole fresh and MP vegetables (Fröder and others 2007; Tresseler and others 2009) have been published but, in general, besides the outbreaks being associated with the consumption of contaminated vegetables it is not possible to identify the pathogen itself implicated in the outbreak (Brasil 2013). Total coliforms higher than 10² MPN/g is commonly reported in those kind of products (Fröder and others 2007; Silva and others 2007; Santos and others 2010), since coliform bacteria occurs naturally on microflora of vegetables (FAO/WHO 2008a). Fecal coliforms detected in RTE lettuce samples reflects unsatisfactory hygienic condition

and did not meet the Brazilian Standards (Brasil 2001) that advocates a maximum of 10^2 /g for fecal coliforms for RTE and MP vegetables. Similar results to those were reported in other studies on fresh lettuce (Loncarevic and others 2005; Fröder and others 2007; Abreu and others 2010; Santos and others 2010). The presence of *E. coli* in whole fresh lettuce could be due to inadequate hygienic practices during preharvest or sporadic contamination through irrigation water (Oliveira and others 2010). *E. coli* prevalence in fresh lettuce found in this study was superior to that reported by others (Loncarevic and others 2005; Oliveira and others 2010). In other studies using MP and RTE vegetables commercialized in Brazil, the occurrence of *E. coli* ranged from 28.6% to 32.9% (Silva and others 2007; Santos and others 2010). As fecal coliforms other than *E. coli*, such as *Enterobacter* and *Klebsiella*, are normally part of native microflora of fresh vegetables, *E. coli* is considered a better indicator of contamination (Loncarevic and others 2005).

The results of microbiological analysis of RTE lettuce served in restaurants indicate deficiencies in hygiene procedures applied during the production line and/or failures in the storage process of these products. The presence of fecal coliforms as well as *E. coli*, suggest that the sanitization of the product was unsuccessful.

Conclusion

Concluding from the results obtained, it seems clear that measures to improve all stages of crop production including irrigation and manipulation of the prepared product should be encouraged, as well as the evaluation of viral detection protocols using new approaches such as PP7 bacteriophage as ICP.

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Author Contributions

M. Brandão carried out the experiments and drafted the manuscript. D. Almeida carried out the experiments and revised the manuscript. F. Bispo carried out the experiments. S. Bricio revised the manuscript. V. Marin revised the manuscript. M. Miagostovich revised the manuscript.

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