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Microbiological and parasitological monitoring in the lettuce production chain of family farming

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Abstract

Lettuce is a vegetable consumed raw and may be a vehicle for diseases caused by microorganisms and parasites. The objective of this work was to carry out microbiological and parasitological monitoring in the production chain of lettuce, determining the main points of contamination during cultivation, harvest, and handling. Microbiological analyses were performed on samples of organic compost, irrigation water, wastewater from the wash tank, water that supplies the wash tank, lettuce, harvester's hands, wash tank surfaces, and harvest boxes, in six production cycles. Parasite analyses were performed on organic compost, water samples, and lettuce. Our results show that the irrigation water proved to be of excellent quality. Nevertheless, the organic compost used for fertilization did not meet the microbiological limits established by current legislation and all samples presented *Entamoeba* spp. cysts. *E. coli* was detected in the hands of the harvester (cycle 3), in the harvest box (cycle 5), and in samples of residual water from the prewash and of the water that supplies the prewash tank. The contamination points detected were not directly related to the contamination of the harvested lettuce. Of the lettuce samples analyzed, only 3% showed unacceptable quality according to current legislation.

Keywords: good agricultural practices, *Escherichia coli*, *Lactuca sativa* L., postharvest

Introduction

Lettuce is a basic item, together with rice and beans, daily present in Brazilian homes, due not only to its nutritional value, but also to Brazilian eating habits. Lettuce (*Lactuca sativa* L.) has the highest commercial value amongst leaf vegetables (Sala & Costa, 2012) but may be an important vehicle of pathogen contamination, as it is consumed raw. In Brazil, 6809 outbreaks of foodborne diseases and 99 deaths were registered between 2009 and 2018. Vegetables represent 2.3% of foodborne disease outbreaks, and the main etiologic agents are *Escherichia coli* and *Salmonela* (Ministério da Saúde, 2020).

In the USA, the Center for Disease Control and Prevention (CDC) investigated an *E. coli* O157:H7 outbreak related to lettuce harvested in the growing region of Salinas Valley, California. Eighty-five out of 167 people infected between September and December 2019 were hospitalized, 15 of whom had hemolytic-uremic syndrome as a result – a type of blood disorder that causes kidney failure. Complete genome sequencing showed that the outbreak was caused by the same strain of *E. coli* O157:H7 that had caused outbreaks linked to leafy greens in 2017 and to romaine lettuce in 2018 (CDC, 2020).

Concerning intestinal parasites in vegetables, there are no official reports, and research is scarce, which results in underestimated data on their prevalence. Nevertheless, lettuce is still the most analyzed vegetable, especially because of the following facts: it goes through an easy production process, it is consumed *in natura*, and it has some chances of contamination through water and soil. For instance, a study conducted in the municipalities of Chapecó and Xanxerê (SC, Brazil), in 2013, analyzed 33 samples of lettuce and showed that 18.18% of them tested positive for *G. duodenalis* cysts (Perdoncini et al.,

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2016). Similarly, Santarém et al. (2012), after analyzing 39 samples of lettuce from establishments in Presidente Prudente (SP, Brazil), identified *G. duodenalis* cysts in 12.8% of the samples (Santarém et al., 2012), whereas Ferreira and co-workers (2020) found *G. duodenalis* in 40% of samples of lettuce, in Pedro Afonso (TO, Brazil) (Ferreira et al., 2020).

Therefore, implementing good agricultural practices in all stages of a production process is important to reduce the possibility of contamination and to ensure food safety (Mattos et al., 2009), as consumers have been more attentive to these issues (Andrade et al., 2013).

In the stage of cultivation, vegetables may be contaminated by enteroparasites and pathogenic microorganisms mainly through irrigation water and/or through contaminated soil due to the use of organic fertilizers containing fecal waste (Abreu et al., 2010; Arbos et al., 2010; Luz et al., 2017). Inadequate handling after harvesting also leads to microbiological contamination of the produce (Lana, 2016).

Having this scenario in mind, we aimed to monitor the production chain of green-leaf lettuce, determining the main points of microbiological and parasitological contamination.

Material and Methods

This study was conducted in a certified organic farm in Agudos (SP, Brazil) (22° 24'E 20" S and 49° 03' 50" W, at a 526-m altitude), where lettuce is cultivated in an arched roof greenhouse with open sides. The crop was fertilized with organic compost produced in the farm, and the sprinkler irrigation system was used. We studied six lettuce production cycles from April 2018 to April 2019 (Table 1) and collected data in different stages:

a) At the beginning of each production cycle: sampling of the organic compost used for fertilization at planting.

b) During the production cycle: sampling of the organic compost used for topdressing fertilization; sampling of the irrigation water; monitoring of temperature and rainfall.

c) At the end of the production cycle: sampling of the harvester's hands; sampling of surfaces of harvest boxes and of wash tanks; sampling of non-washed (directly from the bed) and washed lettuce, of wastewater from washing, and of the water that supplies the tank.

Because the experiment involved a human being through sampling of the harvester's hands, this study was submitted to and approved by the Research Ethics Committee of Centro Universitário Sagrado Coração - UNISAGRADO, Bauru (SP, Brazil) (Approval n. 2.737.238).

Table 1. Description of the production cycles of organic lettuce, Veronica cultivar, including dates of planting and harvest (MM/DD/YEAR), average temperature (°C), and total rainfall (mm).

Cycles	Planting	Harvest	Average temperature (°C)	Rainfall (mm)
1	04/26/2018	06/04/2018	23.1	10.5
2	06/26/2018	08/13/2018	22.1	95.5
3	08/28/2018	10/08/2018	26.3	150.5
4	11/07/2018	12/10/2018	26.9	110
5	01/09/2019	02/11/2019	32.6	180
6	02/27/2019	04/02/2019	29.9	273

Data collection and sampling

Temperature and precipitation: maximum and minimum thermometers (a digital and a liquid-in-glass one) were installed inside the production greenhouse, ~1 m above the ground. A pluviometer was installed on a support post of the greenhouse. All data were collected once a week, during all six production cycles, which generated the average temperature (°C) and total rainfall (mm) in each cycle (Table 1).

Organic compost: we collected portions of the compost in five different points of the pile to ensure a better sampling, resulting in an ~500-g composite sample, which was stored in sterile bags. Two composite samples were collected, one for microbiological analysis and another one for parasitological analysis.

Irrigation water: sampling was carried out directly in the water source (spring), using a 500-mL sterile bottle for microbiological analysis and a 5000-mL one for parasitological analysis. The bottles were stored in an isothermal box and taken to our laboratory for microbiological and parasitological analyses.

Wastewater from tank: for collecting water from the tank after prewashing the lettuce, we sanitized our hands and submerged each previously autoclaved 500-mL bottle (a total of three) in different points in the tank. Subsequently, we attached a 5000-L gallon to the tank's water outlet nozzle to collect a new sample. The bottles were stored in an isothermal box and, together with the gallon, were taken to our laboratory for microbiological and parasitological analyses, respectively.

Tap water that supplies the tank: it comes from the same source as the irrigation water (spring), but it is stored in a water tank. For collection, we opened the tap and allowed the water to flow for 3 min before we collected it using three previously autoclaved 500-mL bottles. The bottles were stored in an isothermal box and taken to our laboratory for microbiological analyses.

Surfaces and harvester's hands: These samples were collected with swabs containing buffered peptone

water. To collect samples from the harvester's hands, we rubbed the swab on the palm of the harvester's hand, under his nails, and between his fingers, always rotating the swab handle on its axis. To collect sample from the inner surfaces of the wash and the water tanks, we rubbed horizontally and vertically two sterile swabs across two 50-cm^2 points, respectively, bounded by a sterile mold. This sampling was carried out in three parts (three repetitions) of the wash and the water tanks each. The samples were stored in an isothermal box and taken to our laboratory for microbiological analyses.

Lettuce: 15 heads of lettuce were harvested, 2/3 of which were washed by immersion in a stainless-steel wash tank. All heads of lettuce were stored in plastic boxes lined with sterile plastic bags and then taken to our laboratory, where we analyzed five non-washed and five washed heads of lettuce. Each head of lettuce represented one repetition. For sampling, we detached two external leaves, two internal leaves, and one central leaf, which were chopped with sanitized utensils and submitted for microbiological analyses. The remaining leaves were submitted for parasitological analyses.

Microbiological analyses of organic compost, surfaces, harvester's hands, lettuce, irrigation water, and tank:

All samples were analyzed for *Salmonella* (presence/absence), total coliforms, thermotolerant coliforms, and *E. coli*. Quantification of mesophiles was also carried out in samples of surfaces, hands, and lettuce. For *Salmonella* analysis, we used dehydrated chromogenic medium, in a ready-to-use Compact Dry®Sl plate (Nissui Pharmaceutical Co.Ltd., Tokyo, Japan), according to the manufacturer's instructions. After pre-enrichment, selective enrichment, inoculation, and incubation at 42ºC for 24 h, we detected a color change of the medium from blue to yellow and the appearance of green or black colonies.

Quantification of total and thermotolerant coliforms and *E. coli* was also performed in dehydrated chromogenic medium in a ready-to-use Compact Dry®Sl plate (Nissui Pharmaceutical Co.Ltd., Tokyo, Japan), according to the manufacturer's instructions. After inoculation and incubation, data were interpreted as described: EC plates enable quantification of total coliforms and *E. coli*. Purple and blue colonies grow in these plates, incubated at 35ºC for 24 h. Blue colonies represent *E. coli*, and the sum of the purple and blue colonies represents total coliforms. Blue colonies, which indicate thermotolerant coliforms, grow in CF plates, incubated at 45ºC for 24 h.

Quantification of mesophiles was performed through surface plating in PCA medium. Plates were incubated at 35ºC for 48 h, following conventional methods (Silva et al., 2010).

For water analysis, we used the membrane filtration technique, with a vacuum filter containing a 0.45-µm sterile membrane. After each filtration, the membrane was placed in Compact Dry plates (Nissui Pharmaceutical Co.Ltd., Tokyo, Japan), previously hydrated with sterile distilled water, according to the manufacturer's instructions.

Parasitological analysis of organic compost, lettuce, and irrigation and tank water:

Compost samples were homogenized one by one, and 10 g was weighted with a precision scale and stored in plastic flasks. To detect helminth eggs and protozoan cysts and oocysts, the compost was homogenized with water and filtered in disposable plastic funnels using a tamis. After filtration, all the material was centrifuged at 2500 rpm for 1 min, in sterile 15-mL Falcon tubes (Sloss, 1999).

Lettuce was analyzed by defoliating the heads individually in plastic recipients, and each leaf was washed using a brush with a solution containing 500 mL of distilled water and 2 mL of neutral detergent. Washing water was filtered in disposable plastic funnels using a tamis covered with gauze pads folded four times. After filtration, all the material was centrifuged at 2500 rpm for 1 min in sterile 15-mL Falcon tubes (Sloss, 1999). After centrifugation, the supernatant was discarded, and the sediment was separated in two halves, one for analysis and the other for the centrifuge-flotation technique with a zinc sulfate solution (Sloss, 1999).

Samples of irrigation water and tank water were distributed in cups for spontaneous sedimentation, for a minimum of 3 h and a maximum of 12 h. The supernatant was discarded, and the sediments were centrifuged at 2500 rpm for 1 min in sterile 15-mL Falcon tubes to concentrate the sediments (Sloss, 1999).

To analyze the sediments obtained from the samples of organic composts, lettuce, and water, ~0.05 mL of the sediment of each sample was used on a glass slide with a coverslip, and Lugol was used as dye for examination under an optical microscope. The slides were analyzed in duplicate (Sloss, 1999).

For analysis of *Cryptosporidium* spp. oocysts in the samples of lettuce, we prepared duplicate slides with the sediment, which were stained through a modified Ziehl-Neelsen technique (Henriksen & Pohlenz, 1981).

Experimental design and result analysis

Experimental design was entirely randomized with five repetitions for lettuce and three for the other points of sampling. For the compost, irrigation water, and harvester's hands, analyses were repeated in triplicate. Results of the microbiological analyses were compared with the tolerance limits stablished by current legislation, when applicable. Samples of hands and surfaces were submitted to analysis of variance, and the means obtained in each cycle were compared by the Tukey test (5%). We used Pearson correlation analysis (5%) to investigate the relation of the incidence of microorganisms on the freshly harvested lettuce to temperature and average precipitation registered in each period evaluated. We also investigated the correlation between contamination of lettuce and the contamination of all elements sampled.

Results and Discussion

Contamination of planting compost and topdressing

Levels of thermotolerant coliforms in the organic compost used as fertilizer in planting and in topdressing revealed to be mostly above the limit (Figure 1) stablished by Brazil's Normative Instruction n. 64, 2008: absence of *Salmonella* in 10 g and a maximum of 3 logCFU. g^{-1} thermotolerant coliforms (Brasil, 2008).

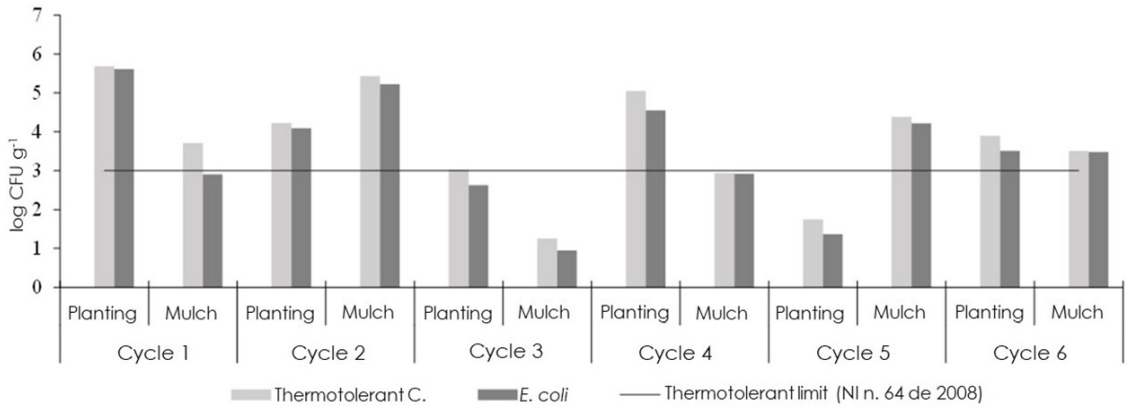

Figure 1. Count of thermotolerant coliforms and *Escherichia coli* in organic compost used as fertilizer in planting and in topdressing. Bauru, Apta Regional, 2019.

The pathogen *Salmonella* was detected only in the planting compost from cycle 1. Levels of thermotolerant coliforms exceeded the limit in most samples, except for the mulch in cycle 3 – in which the farmer used a castor bean-based fertilizer – for the mulch in cycle 4, and for the planting compost in cycle 5 – in which the farmer used matured compost, prepared according to technical orientations based on Kiehl (2012). The bacteria *Escherichia coli* was detected in the compost of all cycles, with counts similar to the thermotolerant coliforms' (Figure 1).

In cycle 4, the material used for topdressing had been composted for 30 days, period corresponding to the initial phase of the maturation process, when levels of thermotolerant coliforms are within the limit determined by legislation. Subsequently, in cycle 5, the same compost was used for planting, having now 70 days of composting and showing a lower level of contamination (Figure 1).

In the property where the experiment was conducted there was not a standard method to prepare the compost. Manure in different production stages and dry matter from diverse sources of raw material were carelessly used, without adjusting them to the correct compost maturation. This is critical for vegetables that are cultivated close to the ground and consumed raw, such as lettuce.

For a correct compost maturation, some processes must occur, such as aeration by constantly mixing and turning the pile over (Kiehl, 2012); however, due to labor shortage, this procedure was not carried out as frequently as needed.

Concerning the presence of parasites in the organic compost, all samples analyzed from the six cycles showed *Entamoeba* spp. cysts. We also detected hookworm eggs and larvae in the compost samples of cycles 1, 2, and 3. It is important to highlight that the presence of hookworm eggs in the compost is directly related to the presence of infected dogs or cats in the properties that cultivate these vegetables (Bowman et al., 2010).

Contamination of irrigation water

Irrigation water had optimal quality, absence of *Salmonella*, and <1 CFU 100 mL^{-1} of thermotolerant coliforms, being in accordance with Brazil's Normative Resolution n. 357, 2005, from CONAMA (Brazil's

Environment Council), which stablishes a limit of 200 thermotolerant coliforms per 100 mL of water in ≥ 80%, from at least six samples collected bimonthly in a year (Brasil, 2005).

Contamination of freshly harvested and prewashed lettuce

Brazil's Normative Instruction (NI) n. 60, from December 23, 2019, determines absence of *Salmonella* in 25 g of *in natura* and whole vegetables and a maximum of 10^3 CFU g^{-1} of *E. coli*. In this NI there is a three-class sampling plan that stablishes a random collection of five sampling units of the same batch to be analyzed individually; these samples are classified into acceptable, intermediate, and inacceptable quality, referring to contamination levels.

In this study all samples had a negative result (absence in 25 g) for *Salmonella*, which is in accordance with the NI.

Regarding *E. coli*, the quality of the non-prewashed and prewashed lettuce revealed to be mostly acceptable (86.7%) amongst the 30 samples collected in the six cycles (Tables 2 and 3), although the organic composts used for fertilization had contamination levels above the limit in most cycles (Figure 1). Pilon et al. (2019) also observed that microbial counts found in fertilizers did not result in similar microbial counts on lettuce.

Table 2. Quantification of samples of non-prewashed lettuce with counts of *Escherichia coli* (*EC*) within the limits established by the three-class sampling plan from NI 60, 2019, and average levels of contamination.

Production cycles	N. of samples within each count limit for *EC* (CFU.g^{-1})[1]			
	up to 10^2	10^2-10^3	>10^3	Average levels (CFU.g^{-1})
	Acceptable quality	Intermediate quality	Inaccetable quality	
1	5	0	0	<10
2	5	0	0	<10
3	4	1	0	8.5 x 10^1
4	4	1	0	1.5 x 10^2
5	3	1	1	8.4x10^2
6	5	0	0	<10
1 to 6	26	3	1	

[1] Colony Forming Unit (CFU.g^{-1}). Bauru, Apta Regional, 2019.

Table 3. Quantification of samples of washed lettuce with counts of *Escherichia coli* (*EC*) within the limits established by the three-class sampling plan from NI 60, 2019, and average levels of contamination.

Production cycles	N. of samples within each count limit for *EC* (CFU.g^{-1})			
	up to 10^2	>10^2-10^3	>10^3	Average levels (CFU.g^{-1})
	Acceptable quality	Intermediate quality	Inaccetable quality	
1	5	0	0	<10
2	5	0	0	<10
3	3	2	0	9.8x10^1
4	4	1	0	1.9x10^2
5	4	1	0	7.8x10^1
6	5	0	0	<10
1 to 6	26	4	0	

[1] Colony Forming Unit (CFU.g-1). Bauru, Apta Regional, 2019.

The number of samples showing intermediate quality in each cycle did not exceed two units, being in accordance with the NI, which determines a maximum of two sampling units with intermediate quality. Only in cycle 5 did one sampling unit of non-prewashed lettuce show inacceptable quality.

The main objective of prewashing is to remove more visible dirt, such as soil, and this should not be a vehicle of contamination. In this study, although the water that supplies the tank was contaminated (Figure 4B, see below), only in cycles 3 and 4 was the average level of *E. coli* higher in washed lettuce than in non-washed lettuce (Tables 2 and 3).

We did not observe a direct relation (p>0.05) between contamination of non-prewashed lettuce and contamination of planting composts and topdressing, with correlation coefficients (r) -0.77 and -0.32, respectively. This can be explained by hygiene habits when harvesting and by the fact that sampling for microbiological analysis was not limited to outer leaves, as they were homogeneously collected from the whole head, including outer, middle, and inner leaves.

Outer leaves are more exposed to environmental conditions and more susceptible to contamination through direct contact with the soil (Bartz et al., 2015). Oliveira et al. (2012) found that *E. Coli* O157: H7 may survive in soil for nine weeks and that bacteria was transferred from soil treated with contaminated compost to the lettuce leaves, mainly the outer ones.

E. coli contamination reached the maximum level of 8.4 x 10^2 CFU.g^{-1} in samples of non-prewashed lettuce (Table 1). These levels of contamination may be reduced to acceptable levels (1.0 x 10^2 CFU.g^{-1}) in samples prepared after correct sanitization. Souza et al. (2018) found a significant reduction in *E. coli* counts (2.5 logarithmic cycles) on lettuce after sanitization with triple vinegar (15g.L^{-1} total acidity expressed as acetic acid); likewise, Lee et al. (2014) observed an ~1-log-cycle reduction in *Escherichia coli* counts on cabbage when sanitized with sodium hypochlorite (200 mg.L^{-1}).

The levels of thermotolerant coliforms were similar to *E. coli*'s, varying from <10 to 1.6 x 10^3 CFU g^{-1}, whereas levels of mesophiles were ~10^6 CFU g^{-1} in all production cycles, in non-prewashed and prewashed lettuce. Mesophiles include deteriorating and pathogenic microorganisms (Silva et al., 2010).

The microorganisms (thermotolerant coliforms, *Escherichia coli*, and mesophiles) quantified in lettuce did not have any relation (p>0.05) to the microorganisms quantified in the harvester's hands, tank surfaces, and harvest boxes, probably because the contact of hands and surfaces are limited to outer leaves; however, this does not mean that hand and surface sanitizations are not necessary.

Parasitological analysis of lettuce samples did not reveal the presence of parasites pathogenic in humans but identified the presence of *Entamoeba* spp. cysts in samples from cycles 1 and 3. Analyses of samples from cycles 2, 4, 5, and 6 were negative. The presence of *Cryptosporidium* spp. oocytes was not detected either.

Average temperature and rainfall and their relation to microorganisms in lettuce

The average counts of mesophiles, thermotolerant coliforms, and *E.* coli were not influenced by environmental conditions (p>0.05); contrarily, Bartz et al. (2015) detected a higher level of contamination in lettuce harvested in the Fall when compared to the Spring, indicating greater survival of pathogens at lower temperatures.

Contamination on the harvester's hands

Total coliforms and mesophiles were detected in the harvester's hands (Figure 2). We observed a significant reduction in total coliforms and mesophiles in cycles 5 and 6 when compared with the others (p<0.05), which shows the importance of hand sanitization, considering that the harvester began to receive instructions on sanitization after cycle 4.

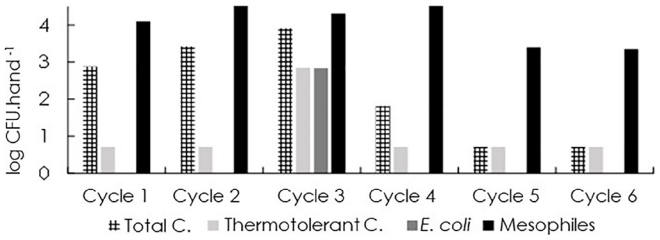

Figure 2. Microorganism counts on harvester's hands. Bauru, Apta Regional, 2019.

Counts of thermotolerant coliforms were lower than the detection limit (detection limit = 5 CFU.$hand^{-1}$ = 0.7 log CFU.$hand^{-1}$), except for cycle 3, in which the values were 2.85 log CFU.$hand^{-1}$ and *E. coli* was detected in levels similar to thermotolerant coliforms' (Figure 1). *Salmonella* was not detected in any of the hand samples.

Contamination of the surfaces of harvest boxes and prewash tank

Mesophiles were the microorganisms mostly found on the surface of the boxes. Their counts in all cycles were >2 log CFU, with no significant differences among the cycles (p>0.05)(Figure 3). *Salmonella* was not detected in any of the samples analyzed, but *E. coli* was detected in samples from cycle 5; this evidences the need of determining better practices to sanitize the harvest boxes, such as to increase the frequency of sanitization and not to place them directly on the ground.

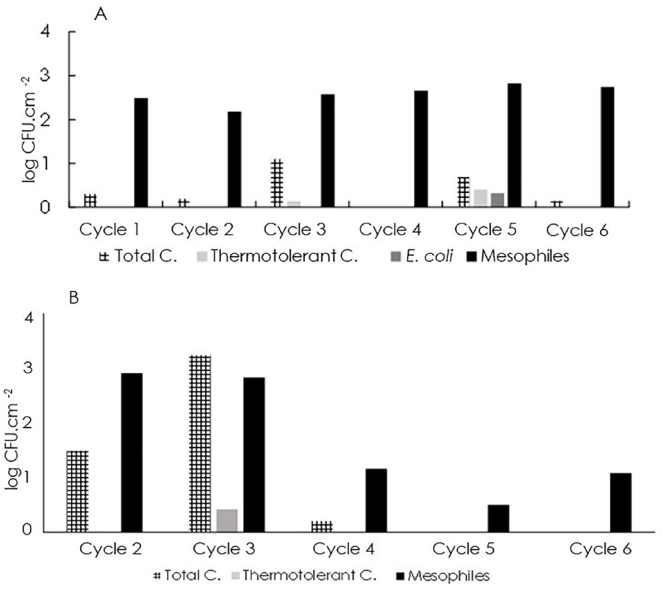

Figure 3. Microorganism counts on the surfaces of boxes (A) and of prewash tanks (B). Bauru, Apta Regional, 2019.

After being harvested, lettuce heads were submerged in water in a stainless-steel tank to remove dirt, such as soil particles and insects. Thus, in samples collected from prewash tanks starting from cycle 2, the results show high levels of total coliforms and mesophiles (Figure 3), which is expected due to visible dirt in the tank.

We observed that tank contamination began to reduce in cycle 4 ($p<0.05$), after the farmer started to sanitize it correctly and routinely: before each use, the farmer washed the tank with sponge and neutral detergent and then sprayed a 200 mg L^{-1} active chlorine solution; this solution remained in the tank for 10 min and was then properly removed with water.

Salmonella was not detected in any of the samples analyzed.

Contamination of residual prewash water

The presence of microorganisms in wastewater from the tank (Figure 4A) is due to the quality of the water that supplies it. After detecting *E. coli* in the wastewater, we suspected that the source water could be contaminated, as it is the same water used for irrigation but stored in a brick reservoir with no appropriate lid. An analysis, in cycle 3, of the water that supplies the tank revealed contamination in levels similar to the wastewater from the tank.

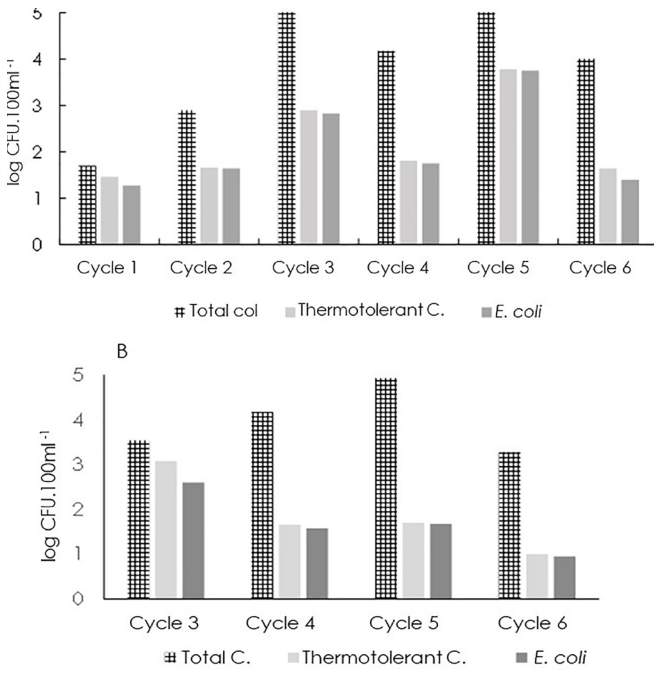

Figure 4. Microorganism counts in residual prewash water (A) and in the water that supplies the prewash tank (B). Bauru, Apta Regional, 2019.

We suggest that brick water tanks should be replaced with vinyl ones and sanitized regularly. Another recommendation is to adjust the farm shed layout. In rainy days, we noticed that raindrops splashed from the roof to the wash tank.

In conclusion, we observed deficiencies in compost preparation and in postharvest procedures, which indicates that the operation, in terms of organization, is below the level recommended for good practices; additionally, we noticed that protocols determined for each operation, from planting to harvest and prewash, were not applied, which is common in family agriculture, as observed by Bartz et al. (2015) in vegetable farms in Southern Brazil.

It is important to highlight that we did not find parasites in the varied water samples analyzed.

Conclusions

The points of contamination detected did not have a direct relation to contamination of the lettuce harvested.

Improvement in the preparation of organic compost and in the procedures of harvest and postharvest is necessary.

Our results, combined with further education (technical courses and field training), will contribute for improvement in the production process, through the implementation of Good Agricultural Practices and Good Postharvest Practices, which ensure that lettuce produced in an organic system is safe and healthy.

Acknowledgments

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