Use of raw and composted poultry litter in lettuce produced under field conditions: microbiological quality and safety assessment

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ABSTRACT Lettuce (*Lactuca sativa* L.) constitutes one the most important vegetable crops worldwide. Poultry litter is being applied as an economically suitable alternative to nitrogen fertilizers in lettuce cultivation. However, little is known about the effects of this practice over this fresh product safety that is usually consumed as a salad. The aim of this work was to determine the microbiological quality and the nitrate content in lettuce produced, under field conditions, using either raw or composted poultry litter, coming from the same original batch. Two experiments were conducted in the experimental field of Facultad de Ciencias Agrarias (UNL, Santa Fe, Argentina) to assess the effects of recently extracted poultry litter that consisted of broiler chicken manure plus rice husk, or composted for 12 mo. The application amounts were: 20 T ha^{-1} (T1); 40 T ha^{-1} (T2); and no application of manure (T). Increasing the applied quantities had also increased the health risk associated with lettuce consumption, due to higher nitrate levels and microbial contamination. However, these risks were reduced by composting the material. Even when lettuce contamination with faecal bacteria was mainly due to the use of poultry litter, the number and incidence of pathogens were reduced when properly composted manure was applied instead of raw one. Increasing the dose of poultry litter applied also increases the health risk in lettuce. Though, when the material is properly composted, its fertilizing capacity is maintained, giving proper yields with lower nitrate levels and microbial contamination by enterobacteria.

Key words: lettuce (*Lactuca sativa* L.), microbial contamination, nitrate levels, compost, poultry litter, food safety

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INTRODUCTION

Lettuce (*Lactuca sativa* L.) is a leafy herbaceous self-pollinated annual plant of the Asteraceae family. It is cultivated worldwide and usually consumed as a green salad. Lettuce (and chicory) estimated global production was nearly 25 million T in 2014, as was informed by the Food and Agriculture Organization of the United Nations (Armas et al., 2017). Despite this huge amount of lettuce harvested, its production and the production of other vegetables must be constantly enlarged to keep up with the demand of a rapidly increasing world population. This yield improvement is usually achieved by applying nitrogen fertilizers, because the scarcity of this element most commonly limits plant growth. Nevertheless, the use of large amounts of nitrogen is expensive and could contaminate surface and ground waters (Barrameda-Medina et al., 2017).

In terms of volume, litter represents the main solid leftover from primary level in different poultry industry (Vaz et al., 2017). Poultry litter is being applied as an economically suitable alternative to chemical fertilizers. Mainly, the use of composted poultry manure allows the recycling of organic material and nutrients and also adds value to this waste product (Caceres et al., 2015). The main problem about this practice is the threat of spreading manure-borne pathogenic microorganisms, mostly when is applied to vegetables, which will then be consumed as fresh products (Marti et al., 2013). This risk increases with the use of raw manure, in particular poultry litter, a usual practice in vegetable production (Rotondo et al., 2009). The use of composted manure can reduce microbiological contamination, particularly with enteropathogenic bacteria (Erickson et al., 2015), since aerobic composting of animal manure is a beneficial treatment that inactivates these pathogens (Erickson et al., 2014).

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Leafy green vegetables, including lettuce, are recognized as potential vehicles for foodborne pathogens such as *Escherichia coli* O157: H7 (Pang et al., 2017) and *Salmonella spp.* (Velasquez et al., 2018). The transfer of pathogens is presumed to occur largely through direct contact of aerial tissue with the ground or through rain or irrigated water splashes of soil onto the aerial tissue (Allende et al., 2017). As *E. coli* is the universal indicator of faecal contamination, it has became the ideal marker in the microbiological analysis of fresh foods and, particularly, in leafy vegetables such as lettuce (Ceuppens et al., 2014).

Besides the risk of microbiological contamination, in the recent years, excess nitrate intake has also increased in parallel with increasing consumption of fresh fruits and vegetables (Gil et al., 2015). Accumulation of nitrate in plants depends on several factors, both genetic and environmental, although leafy vegetables, such as lettuce or spinach, contain the highest concentrations (Iammarino et al., 2014). A factor that would increase nitrate concentration is the use of raw manure, instead of composted one, in vegetables production (Biala et al., 2016).

Accumulation of high amounts of nitrates in leafy vegetables can be toxic for humans due to its impact on the occurrence of certain diseases, such as methaemoglobinemia in children (Dellavalle et al., 2013), and because nitrate, as nitrosamine precursor, is a potential carcinogen (Jaworska, 2005). However, despite these facts, a dilemma began to pose recently about their possible beneficial effect on human health, particularly, on the reduction of blood pressure (Alissa and Ferns, 2017). It must be kept in mind that, approximately, 80 % of dietary nitrates are derived from vegetable consumption (Brkic et al., 2017).

The aim of the present study was to determine the microbiological quality and nitrate content in lettuce produced, under field conditions, using either raw or composted poultry litter, coming from the same original batch.

MATERIALS AND METHODS

Description of the Experimental Field Site

The studies were conducted at the experimental field of Facultad de Ciencias Agrarias (Universidad Nacional del Litoral, Santa Fe, Argentina, latitude 31° 25′ south, 60° 56′ west) during the autumn growing seasons of 2 successive years. Climate is a subhumid–humid mesodermic (C2B‘3rd’), according to Thornthwaite (1948) with an annual precipitation of 1000 mm approximately. The site consisted of a 0.2-ha piece of land that was quite flat and was previously used for growing a variety of vegetable crops but without the use of organic manure. The soil was typical Argiudoll Esperanza Serie, which chemical characterization, performed before the beginning of this study on samples taken up to 0.20 m, was as follows: organic matter 2.2 %; total nitrogen 0.112 %; phosphorus 54 ppm (Bray and Kurtz, 1945); pH 6.8 (1:2.5); electrical conductivity 0.6 dS/m; calcium, magnesium, sodium, potassium, and cation exchange capacity 10.2; 2.1; 1.0; 1.4 and 14.8 cmol kg^{-1}, respectively.

Experimental Design

Two experiments were performed in successive years, 2016 and 2017, from February to May. In the first one (2016), poultry litter recently removed from the broiler house was directly applied to the soil. A part of this same material was composted for 12 mo, according to the procedure described below, in order to use it in the second experiment (2017). Poultry litter was incorporated to the soil at a depth of 10 cm previously to transplantation. In order to avoid possible interference between experiments, these were carried out at different sites 30 m apart from each other.

In both experiments, crop management practices were the same. Lettuce seedlings cv. “Brisa” were 30 and 28 d old when they were transplanted into the field in 2016 and 2017, respectively. The cultivation was made in double row, leaving 0.35 m between plants, with a density of 8.0 plants m^{-2}. Harvest was carried out 50 d after the transplant to determine yield.

In these 2 assays, three treatments were performed: without (T), with 2 kg m^{-2} (T1), and with 4 kg m^{-2} (T2) of poultry litter, respectively. The experimental design was complete randomized, of 3 treatments and 4 replicates, making a total of twelve experimental units. Each experimental unit consisted of plots that included 3 rows of 0.7 m apart and 5.0 m long. Samples were taken from plants in the central row of each treatment plot and carefully manipulated to avoid cross-contamination.

Poultry Litter Composition and Composting Method Description

The poultry litter used consisted of broiler chicken manure plus rice husk that had accumulated in the broiler house for about 25 wk. The litter contained about 46 % moisture; pH 7.7 (1:2.5); electrical conductivity 6.2 dS m^{-1}; density 0.4 g cm^{-3}; N-NH_4 content 59 mg kg^{-1}; total nitrogen 15.6 g kg^{-1} on a dry weight basis; carbon: nitrogen ratio 18.2, and a bacterial load of 40 000 CFU g^{-1}.

The aerobic composting was performed as was outlined by Iglesias-Jiménez et al. (2008). Briefly, poultry litter was piled on the ground and, twice a month, the pile material was lifted and mixed with a cradle to avoid an excessive rise of temperature. Compost was considered mature when the original material could not be distinguished.

Nitrate Analysis

Nitrate concentration (ppm) was determined in the youngest fully expanded leaves harvested before noon (Siomos, 2000), using a nitrate ion meter, N-NO_3 (Horiba Cardy Meter). The concentration of sap nitrate (ppm) was then converted to mg kg^{-1} considering a 6 % of dry matter. The determination of sap nitrate concentration was performed with an error of 1%.

Total Coliforms, faecal Coliforms and E. coli Determinations

A day before the beginning of the harvest, samples were taken consisting in 5 basal leaves of the plants for each repetition, taking care that they would not have had a direct contact with the ground. Samples were introduced into bags, that were subsequently sealed, labeled and immediately transported to the Food Analysis Laboratory (Departamento de Salud Pública, Facultad de Ciencias Veterinarias, UNL), where 10 g (fresh weight) were taken and introduced in an Erlenmeyer flask with 90 mL of buffered peptone water.

Once the sample was processed, the count of Coliforms was assessed using the 3-tube most probable number (**MPN**) technique (Teramura et al., 2017), as resumed next:

1) A total of 9 tubes per sample, containing 10 mL of Mac Conkey media (Britania, Argentina), were prepared. Out of them, 3 were prepared with double concentration of the media (2X). All tubes were sterilized for 15 min at a pressure of 1 atm in an autoclave.
2) Then, inoculation was carried out as follows: 3 tubes with 10.0 mL of sample (media at 2X), 3 with 1.0 mL, and the rest with 0.1 mL.
3) The tubes were incubated at 37°C for 48 h.
4) After this incubation period, the tubes were analyzed to assess the presence of coliforms, considering as "positive" those that showed the presence of gas, turbidity and turned yellow, and as "negative" those that did not showed these parameters or only one of them. Once the profile of positive and negative tubes was established, the MPN table was used to obtain the number of microorganisms found in the sample.
5) A small aliquot was taken from all the positive tubes and each was added to another tube with fresh McConkey media. Then, tubes were incubated at 44°C for 48 h to check the presence of faecal coliforms.
6) "Positives" were grown in sterilized Petri dishes with EMB Agar media (Teramura et al., 2017) and were incubated at 44°C for 48 h.
7) Finally, results interpretation was carried out to identify the presence ("positive") or absence ("negative") of *E. coli* in the samples analyzed. Samples were considered as "positive" when they had a metallic shine and "negative" otherwise.

Then, 4 complementary biochemical tests were performed to verify the presence of this specific pathogen, which included the inoculation of positive samples in four different media: Citrate, TSI, Phenylalanine, and SIM (Teramura et al., 2017).

Results are expressed as CFU g of $lettuce^{-1}$.

Statistical Analysis

The corresponding statistical model for these experiments was $y_{ij} = \mu + \alpha_i + E_{ij}$, where y_{ij} is the dependent variable analyzed (lettuce total yield, lettuce nitrate concentrations, and number of total coliforms, faecal coliforms and *E. coli*), α_i is the fixed effect of i treatment (effect of poultry litter T, T1, and T2 treatments, non-composted in 2016 and composted in 2017), and E_{ij} is the random error associated with the j_{th} observation in treatment i. Statistical differences were determined between treatments, in each year, by one way ANOVA and Tukey's mean separation test for multiple comparison. Because the use of the same starting poultry litter batch in both experiments was prioritized, instead of using different materials in the same year, the comparison between years cannot be included. Errors in the statistical models were checked and passed normal distribution with constant variance. Results are expressed as mean ± SE (n = 4). For all analysis, significance was determined at $P \leq 0.05$.

RESULTS AND DISCUSSION

Agricultural production must be rapidly enlarged to meet the food demand of a constantly increasing world population. Yield rises can be achieved by supplying crops with all they need to grow, mainly a nitrogen source. Poultry litter is being applied as an alternative of lower cost to chemical nitrogen fertilizers, even in the production of fresh vegetables as lettuce. However, there is a dearth of information pertaining to the use of poultry litter on this fresh product safety that is usually consumed as a salad.

In our experiments, we have applied different amounts of poultry litter (none in treatment T; 2 kg m^{-2} in T1, and 4 kg m^{-2} in T2), either raw (year 2016) or composted for 12 mo (year 2017). Regardless of the amount applied, the yields measured were greater in the first year (2016) compared to the second one (2017). This difference could be due to the greater number of cloudless days; nevertheless this does not affect the comparison between treatments within each year.

In both years (2016 and 2017), the highest yield was obtained using 4 kg m^{-2} of poultry litter (T2), reaching values significantly higher than when no manure was applied (T) (year 2016: 3.8 ± 0.3 vs 2.5 ± 0.05 kg m^{-2}; year 2017: 3.3 ± 0.15 vs 2.1 ± 0.05 kg m^{-2}, respectively,

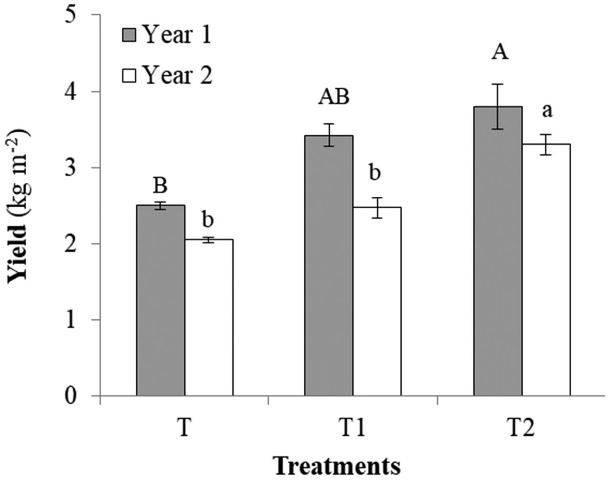

Figure 1. Effect of the treatments performed with raw (year 1, grey bars) and composted (year 2, white bars) poultry litter on lettuce total yield: without (T), and with 2 kg m^{-2} (T1) and 4 kg m^{-2} (T2) of poultry litter. Within the same year, means with different letters are significantly different (n = 4, Tukey´s test, P < 0.05). The lines on bars indicate standard error.

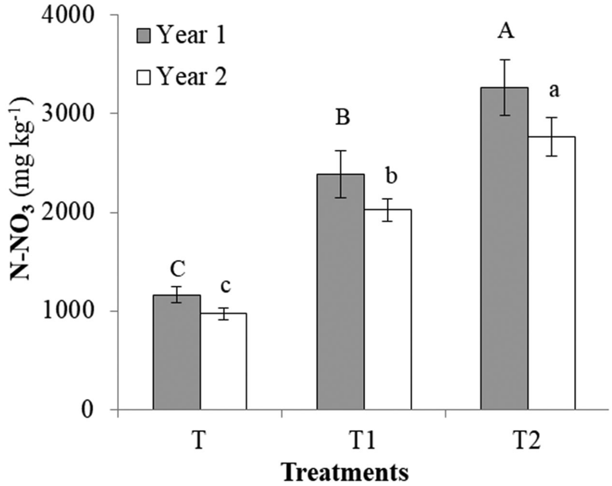

Figure 2. Effects of the treatments performed with raw (year 1, grey bars) and composted (year 2, white bars) poultry litter on lettuce nitrate concentrations (mg kg^{-1}) after 50 d of cultivation. Within the same year means with different letters are significantly different (n = 4, Tukey´s test, P < 0.05). The lines on bars indicate standard error.

n = 4, $P < 0.05$, see Figure 1). In comparison with other works, these level of manure resulted in an intermediate value for this crop (Masarirambi et al., 2012). A direct correlation between fertilizer dose and lettuce yield can be appreciated: higher doses have given higher yields (see Figure 1) though in the first year the differences between T1 (3.4 ± 0.15 kg m^{-2}, n = 4) and the other treatments were not statistically significant. Also, the results achieved correlate with the effect of raw poultry manure on lettuce growth and yield previously studied by Lim (2016), who has obtained a yield response represented by the equation $Y = -0.0067x^2 + 0.6239x + 6.6947$, where x is the applied manure dose. The optimum poultry manure dose determined by Lim (2016) was 46.6 T ha^{-1} (or 4.66 kg m^{-2}) and resulted in a yield of 21.2 T ha^{-1} (or 2.12 kg m^{-2}). Even when this optimum dose gives a value slightly higher than the maximum used in this work (4.0 kg m^{-2}, T2), the yields measured in our experiments for this amount of manure were 3.8 ± 0.3 kg m^{-2} when using raw poultry litter (year 2016) and 3.3 ± 0.15 kg m^{-2} for composted one (year 2017), values higher than those obtained by Lim (2016), supporting our results.

Besides the highest yield, T2 treatment has also given the highest lettuce nitrate concentration in both years: 3263 ± 279.2 mg kg^{-1} in 2016 and 2764 ± 197.5 mg kg^{-1} in 2017 (see Figure 2). Moreover, the value obtained during the year 2016, where raw poultry litter (without compost) was used, surpassed the 3000 mg kg^{-1} (Figure 2). The European Economic Community stated this value as the maximum admissible in lettuce grown in open air in a season similar to this experiment (EEC, 2011). Therefore, the use of raw poultry litter at a dose of 4.0 kg m^{-2} it may not be advisable for production conditions similar to the ones used in this experiment. Instead, regarding lettuce nitrate levels, composted poultry litter could be applied even in this high concentration.

Also, it must be considered that the European Communities Scientific Committee for Food (SCF) set the acceptable daily intake of NO_3^- at 3.65 mg kg^{-1} body weight day^{-1} (SCF, 1992). From this value, it can be inferred that the nitrate daily intake of a person weighing 70 kg should not exceed 256 mg. This limit will be surpassed if this person eats a salad containing more than 70.7 g of lettuce fertilized with 4.0 kg m^{-2} (T2) of raw poultry litter (100 g of lettuce contain 362.3 mg of nitrate), while for lettuce cultivated with composted poultry litter, this limit will be exceeded eating more than 92.6 g (100 g of lettuce contain 276.4 mg of nitrate). The above exposed results show the importance of the topic for food security, indicating that a person could safely consume a 31% more of lettuce if it is fertilized with composted poultry litter.

A positive correlation between plant nitrates and applied nitrogen was determined by McCall and Willumsen (1999). In this work, we also observed this positive correlation (see Figure 2); however, the nitrate concentration increasing rate was lower using composted poultry litter (year 2), what can be attributed to the lower mineralization rate of the already stabilized compost (Garcia-Gomez et al., 2003). In raw poultry litter (year 1), ammonium is rapidly oxidized and nitrogen became available as nitrate for crop uptake (Pino et al., 2008).

As was already mentioned, foodborne diseases constitute one of the most widespread health problems. In the last years, the rise in the consumption of fresh fruits and vegetables has increased not only the risk of excess nitrate intake but also the threat of developing diseases caused by pathogenic microorganisms carried by these products (Gil et al., 2015). Therefore, in this work, we have paid special attention to determine the microbial content in lettuce produced using raw or

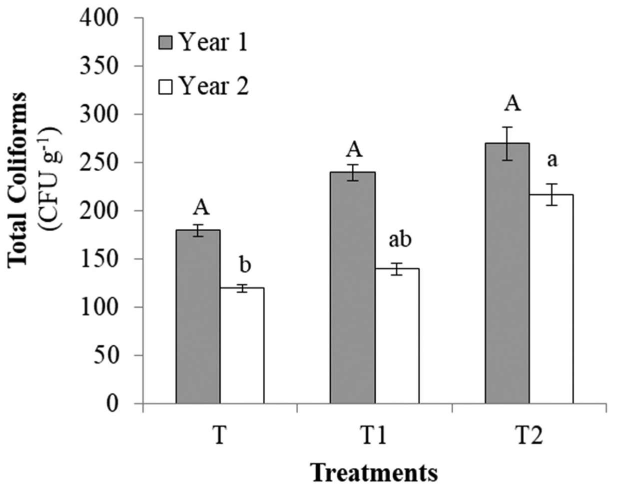

Figure 3. Effects of the treatments performed with raw (year 1, grey bars) and composted (year 2, white bars) poultry litter on the total coliforms, expressed in Most Probable Number (MPN per 100 mL), in lettuce after 50 days of cultivation. Within the same year means with different letters are significantly different (n = 4, Tukey´s test, $P < 0.05$). The lines on bars indicate standard error.

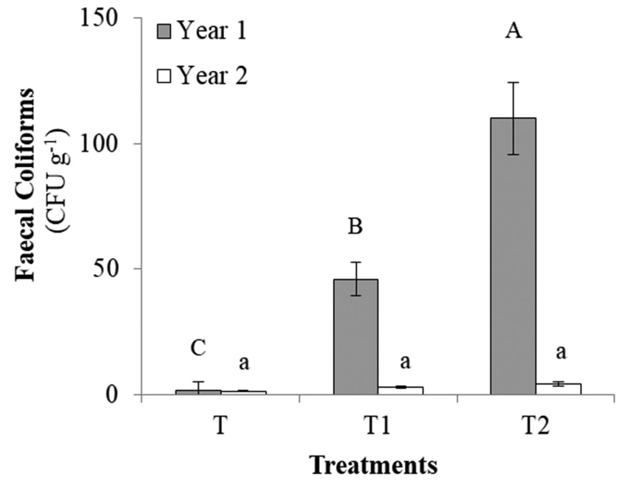

Figure 4. Effects of the treatments performed with raw (year 1, grey bars) and composted (year 2, white bars) poultry litter on the faecal coliforms, expressed in Most Probable Number (MPN per 100 mL), in lettuce after 50 d of cultivation. Within the same year means with different letters are significantly different (n = 4, Tukey´s test, $P < 0.05$). The lines on bars indicate standard error.

composted poultry litter. When comparing the amount of total coliforms in lettuce samples, no differences were found between treatments within the first year (see Figure 3). However, in the second year, the lowest value corresponded to T treatment (120 ± 4.2 CFU g^{-1} of lettuce), although no differences were found with T1 (140 ± 6 CFU g^{-1} of lettuce) or between T1 and T2 (218 ± 12.1 CFU g^{-1} of lettuce, n = 4, Figure 3). It must be taken into account that total coliforms are not necessarily indicators of faecal contamination because coliforms are naturally found in soil as environmental coliforms. Regarding this subject, Giddens and Barnett (1980) studied the total coliform bacterial content in the runoff surface of soils amended with poultry manure. They found no relationship between the application dose and the number of total coliforms in the runoff water, when applied to fallow soil.

Faecal coliforms constitute a subgroup within total coliforms that can be distinguished by their ability to grow at elevated temperatures. They are associated with the faecal material of warm-blooded animals and primarily include *E. coli* and some strains of *Enterobacter* and *Klebsiella* (Martin et al., 2016). Unlike total coliforms, faecal coliforms contamination of lettuce plants suffered a statistically significant increase with increasing applied doses of raw poultry litter (1.9 ± 3.4; 46.9 ± 6.75, and 110.0 ± 14.4 CFU g^{-1} of lettuce for T, T1, and T2 treatments, respectively, n = 4, $P < 0.05$, Year 1, Figure 4). In contraposition, the number of faecal coliforms is drastically diminished when composted poultry litter is applied, and no difference is observed between treatments (1.3 ± 0.2; 3.1 ± 0.4, and 4.2 ± 0.8 CFU g^{-1} of lettuce for T, T1, and T2 treatments, respectively, Year 2, Figure 4). These results demonstrate, on one hand, that poultry litter is a source of pathogens that can pollute the environment or contaminate fresh products (Aruscavage et al., 2006), so it could be associated with foodborne outbreaks (Chen and Jiang, 2014). On the other hand, that the use of properly composted poultry manure reduces the number and incidence of pathogens (Erickson et al., 2014). In this regard, our results agreed with the outcome of other studies performed indicating that aerobic composting of animal manure is a beneficial treatment that inactivates these pathogens (Chen and Jiang, 2014). Nonetheless, it must be considered that some pathogenic cells could survived the composting process and persist in compost-amended soils (Lemunier et al., 2005), what could lead to the threat of pathogen regrowth (Kim et al., 2009).

In order to go deeper into the microbiological analysis, we have determined the presence of *E. coli*, the universal indicator of faecal contamination, in lettuce plants produced using raw or composted poultry litter. As can be appreciated in Figure 5, in both cases, microbiological contamination with *E. coli* was higher in T1 and T2 treatments in comparison with the use of no manure (T) (Year 1: 0.3 ± 0.05; 4.3 ± 0.5, and 5.3 ± 0.6 CFU g^{-1} of lettuce for T, T1, and T2 treatments, respectively, n = 4, $P < 0.05$; Year 2: 0.5 ± 0.1; 1.8 ± 0.3, and 2.6 ± 0.3 CFU g^{-1} of lettuce for T, T1, and T2 treatments, respectively, n = 4, $P < 0.05$, see Figure 5). Also, it can be noted in Figure 5 that the incidence of contamination with *E. coli* was reduced when composted poultry litter was applied. These results, obtained in lettuce produced under field growing conditions, agreed with the fact that composting is a recommended practice to disinfect the manure and reduce microbial contamination of vegetables and other produces during the production stage (Marti et al., 2013). Although the amount of *E. coli* found in lettuce is very low, it can be risky for public health. Therefore, cleaning and disinfection procedures during the preparation of raw vegetables should be very careful to prevent *E. coli* from entering the consumer's plate.

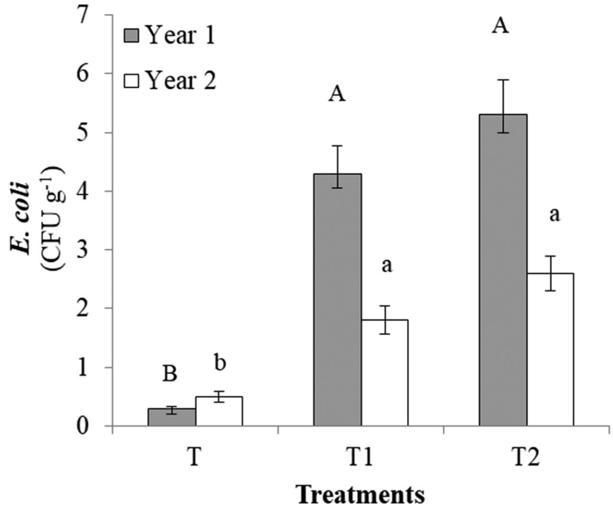

Figure 5. Effects of the treatments performed with raw (year 1, grey bars) and composted (year 2, white bars) poultry litter on the population of *E. coli*, expressed in Most Probable Number (MPN per 100 mL), in lettuce after 50 d of cultivation. Within the same year means with different letters are significantly different (n = 4, Tukey´s test, $P < 0.05$). Bars indicate standard error.

Finally, when comparing the amounts determined, in lettuce samples, for total coliforms (Figure 3) and faecal coliforms (Figure 4), the diminution observed in CFU g^{-1} of lettuce could be due to the existence of several genera of coliforms that are common contaminants of non-faecal sources, e.g., *Klebsiella, Enterobacter*, and *Citrobacter* species (Ceuppens et al., 2014). On the other hand, there is also a decrease between the amount of faecal coliforms (Figure 4) and the amount of *E. coli* (Figure 5) determined. These results may be relevant for lettuce management practices, considering that *E. coli* is the only valid indicator of fresh produce faecal contamination (Martin et al., 2016).

Although it was not studied here, some evidences indicate that the mechanism by which pathogens are introduced into lettuce plants is favored by plant contamination when it is grown in fields fertilized with improperly treated manure (Allende et al., 2017).

In summary, our results showed that increasing the dose of poultry litter applied also increases the health risk in lettuce. Though, when the material is properly composted, its fertilizing capacity is maintained, giving proper yields with lower nitrate levels and microbial contamination by enterobacteria.

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