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Suitability of centrifuge water for detecting the presence of *Escherichia coli* versus finished fresh-cut lettuce testing

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ABSTRACT

Fresh produce causes most foodborne outbreaks in the USA, and it is also considered a hazardous food product in other areas of the world such as Europe. The outbreaks attributed to fresh produce increase the focus of producers on hygiene to minimize exposure to food hazards. The fresh produce industry has the urgent need to detect if there are production lots contaminated with pathogenic microorganisms before distribution. Although the industry is mostly using end-product testing for the detection of target microorganisms, previous studies have evaluated the suitability of different sampling points within the production line of a fresh-cut processing plant. In the present study, the centrifuge effluent water was assessed as an alternative sampling point to end-product testing. *E. coli* was selected as an index microorganism of the presence of pathogens. The presence of *E. coli* was assessed in centrifuge effluent water, and fresh-cut lettuce from a commercial fresh-cut produce processing line (n = 95). The rate of false positives and negatives, as well as the specificity, sensitivity, and efficiency of the alternative method were calculated. The mean population of *E. coli* in positive water samples was 0.86 log cfu/100 mL, while the mean population of *E. coli* in positive fresh-cut lettuce samples was 0.23 log cfu/g. The proportion of positive samples in centrifuge effluent water and lettuce was similar (≈20%), and most of the results in both matrices were coincident (81.1%). However, the alternative method was not reliable due to its low sensitivity, as only 47.6% of the lettuce samples positive for *E. coli* could be matched with positive water samples.

1. Introduction

The microbiological safety of fresh produce is being acknowledged as an increasing health risk concern due to the growing consumption of these products, and the improvement in pathogen detection methods Jacxsens et al. (2017). Determining the microbiological safety status of fresh produce remains a challenge due to the sporadic and heterogeneous occurrence of the target microorganisms, and the difficulties in their detection (Castro-Ibáñez et al., 2016). The study of Pérez-Rodríguez et al. (2014) showed that, due to the distribution of microbial contamination in produce, reliable sampling plans based on direct produce analysis are not feasible. However, the fresh produce industry, as well as government agencies, still use end-product analysis to test for microbial contamination in food.

In an attempt to solve the problems linked to the distribution pattern of pathogens in fresh produce, the analysis of process water has been proposed as a potential alternative to the direct examination of produce (Gil et al., 2009; Magaña et al., 2014). In the case of sprouted seeds, current European legislation (EC, 2013) specifies that it is appropriate to provide alternatives to the sampling of sprouts in cases where the sampling is technically challenging. They suggest the testing of spent irrigation water for pathogenic bacteria as an alternative strategy, as it seems to be a good sampling point for index microorganisms in the sprouts themselves. However, due to the uncertainties regarding the sensitivity of this strategy, the legislation indicates that food companies using this alternative should establish a sampling plan, including sampling points for spent irrigation water.

In the case of fresh and fresh-cut leafy vegetables, the analysis of the centrifuge effluent water has been proposed as an alternative strategy to the sampling of fresh produce for the detection of microorganisms (Buchholz et al., 2012; Tomás-Callejas et al., 2012; Castro-Ibáñez et al., 2016). However, these claims are based on lab-scale studies using artificial inoculation of fresh produce. The work by Gomez et al. (2010) showed that centrifugation water could help in estimating the microbial load of fresh-cut lettuce. In the study by Castro-Ibáñez et al. (2016), apart from the lab-scale tests, samples from produce, water, and

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surfaces from fresh-cut produce processing companies were analyzed. Positives for *E. coli* and confirmed positives for *Salmonella* spp. were only found in the centrifuge effluent water.

The present study aimed to assess the analysis of centrifuge effluent water as an alternative method to end-product testing for the detection of target microorganisms in a fresh-cut produce processing plant. The detection of the index microorganism *E. coli* in fresh-cut iceberg lettuce was used as a model. The impact of temporal variability, including both seasonal and daily variability, in the number of samples positive for *E. coli* was evaluated. The aim was to assess whether temporal trends could be identified and correlated with an increase in the prevalence of *E. coli* in the centrifuge effluent.

2. Materials and methods

2.1. Experimental design

Centrifuge effluent water and produce samplings were performed at a fresh-cut produce facility located in Murcia (southeastern Spain). The sampled line processed fresh-cut iceberg lettuce. Sodium hypochlorite was used as a processing aid to maintain a low microbial load in the wash water during washing. The product throughput varied between 1200 and 2400 kg/h in the studied line. The processing line was equipped with two horizontal centrifuges working in parallel. Effluent from both centrifuges was evacuated through a stainless steel canal to a shared drain. Six independent samplings were performed in six different days between June and September 2018. During each of the six samplings, 10 to 20 samples of centrifuge effluent water and fresh-cut iceberg lettuce were collected and quantitatively analyzed for *E. coli*. In all, 95 centrifuge effluent water and fresh-cut iceberg lettuce samples were examined. Table 1 shows the number of water and lettuce samples taken per sampling day.

2.2. Water sampling and analysis

The sampling period in the fresh produce processing plant was from 8:15 to 13:30 h. Within this period, every 30–60 min, 2-L samples of centrifuge effluent water were collected from the drain of the two centrifuges in sterile plastic bottles (Deltalab, Barcelona, Spain). The concentration of free chlorine in centrifuge effluent samples was measured using chlorine test strips (Lamotte, Chestertown, USA). Sodium thiosulphate (0.05 M) was added to the water samples to quench free chlorine residuals. At the end of the sampling period, samples were transported to the laboratory in a cool box and stored (< 24 h) under refrigeration (4 °C) before further analysis.

In the laboratory, an aliquot (100 mL) from each sample was transferred to three sterile tubes (~34 mL per tube) to concentrate the samples by centrifugation at 4500 × *g* for 10 min at 4 °C (Sobsey, 1999). The suitability of the centrifugation method for the concentration of *E. coli* in the samples was confirmed by preliminary tests using centrifuge water inoculated with different levels of *E. coli*. The supernatant was discarded, and the pellets from the three tubes were sequentially resuspended in 1.1 mL of buffered peptone water (BPW, 2 g/L). Samples were then plated in Chromocult coliform agar (Merck, Darmstadt, Germany) and incubated at 37 °C for 24 h. Dark-blue to violet colonies were considered positive for *E. coli*. The detection limit for *E. coli* in water samples was 1 cfu/100 mL (0 log cfu/100 mL).

The physicochemical analysis included Chemical Oxygen Demand (COD), turbidity, oxidation-reduction potential (ORP), pH, and conductivity. The standard photometric method (APHA, 1998) using a photometer (Spectroquant NOVA 60, Merck) was used to determine COD. Turbidity was measured using the turbidimeter Turbiquant 3000 IR (Merck, Darmstadt, Germany). ORP and pH were measured using a multimeter pH and Redox 26 (Crison, Barcelona, Spain). Conductivity was assessed using a conductivity meter CM35 (Crison, Barcelona, Spain).

Table 1
Sampling date and the number of centrifuge effluent water and fresh-cut lettuce samples obtained from the six different samplings performed at the fresh produce processing plant.

Sampling	Date	Water samples	Fresh-cut lettuce samples
1	June, 28	18	18
2	July, 26	20	20
3	August, 16	20	20
4	August, 23	20	20
5	September, 13	10	10
6	September, 27	7	7
Total		**95**	**95**

2.3. Fresh-cut lettuce sampling and analysis

Every 30 min, two samples (300 g each) of fresh-cut lettuce were collected from the conveyor belts that transport the dewatered lettuce from the centrifuge to the packaging area. Disinfected gloves were used to place the samples in sterile plastic bags. The lettuce samples corresponded to the lot that was centrifuged when the water samples were taken, as the goal was to link the presence of *E. coli* in the centrifuge effluent water with its presence in the produce. Samples were transported to the lab in cool boxes, and stored overnight under refrigeration (4 °C) at the end of the sampling day before further processing. Once in the laboratory, 25 g aliquots were diluted 1:5 in BPW (20 g/L) and homogenized in a stomacher for 1 min. From the homogenate, 1 mL was pour plated (Castro-Ibáñez et al., 2016). In both cases, Chromocult and incubation at 37 °C for 24 h was used for the plating. Dark-blue to violet colonies were considered positive for *E. coli*. The detection limit for lettuce samples was 5 cfu/g (0.7 log cfu/g).

2.4. Data analysis

R software (R Development Core Team, 2017) was used for statistical analysis. Centrifuge effluent water was assessed as an alternative strategy for the sampling of fresh-cut products. The result obtained at the same sampling time using both approaches (centrifuge effluent water and fresh produce) was compared. The results were categorized as follows: True positive (TP): positive for *E. coli* in water and in produce; False positive (FP): positive for *E. coli* in water when negative in produce; True negative (TN): negative for *E. coli* in water and in produce; False negative (FN): negative for *E. coli* in water when positive in produce. Some parameters (false positives rate, false negatives rate, sensitivity, specificity, and efficiency) were calculated to assess the alternative strategy. False positives rate is the probability of having a positive result in the water when the lettuce samples are negative (Eq. (1)). False negatives rate is the probability of having a negative result in the water when the lettuce samples are positive (Eq. (2)). Sensitivity is the capability for detecting positive samples in the water when the lettuce sample is positive (Eq. (3)). Specificity is the capability for detecting negative samples in the water when the lettuce sample is negative (Eq. (4)). Efficiency would be the percentage of right guesses using the results of water to predict the result in lettuce (Eq. (5)).

$$FP\ rate = \frac{Number\ of\ positive\ water\ samples\ when\ lettuce\ is\ negative}{Number\ of\ negative\ lettuce\ samples} \cdot 100 \quad (1)$$

$$FN\ rate = \frac{Number\ of\ negative\ water\ samples\ when\ lettuce\ is\ positive}{Number\ of\ positive\ lettuce\ samples} \cdot 100 \quad (2)$$

$$Sensitivity = \frac{Number\ of\ positive\ water\ samples\ when\ lettuce\ is\ positive}{Number\ of\ positive\ lettuce\ samples} \cdot 100 \quad (3)$$

$$Specificity = \frac{Number\ of\ negative\ water\ samples\ when\ lettuce\ is\ negative}{Number\ of\ negative\ lettuce\ samples} \cdot 100 \quad (4)$$

$$Efficiency = \frac{Number\ of\ samples\ with\ a\ coincident\ result\ in\ water\ and\ lettuce}{Total\ number\ of\ samples} \cdot 100 \quad (5)$$

Additionally, the temporal variability in the number of samples positive for *E. coli* was assessed. The influence of the sampling month and the time of day when the samples were taken was assessed. Regarding the number of positive samples by sampling month, June, July, August, and September were compared. To study the number of positives depending on the time of day, the sampling period (8:15–13:30 h) was divided in six time slots: 8:15–9:00 h, 9:00–10:00 h, 10:00–11:00 h, 11:00–12:00 h, 12:00–13:00 h, and 13:00–13:30 h. As the number of samples analyzed in these different time slots and months was not the same, a factor was calculated to allow direct comparison. The percentage of the total number of positive samples detected at a specific time slot or month was divided by the percentage of the total number of samples taken at the same time slot or month to calculate the factor.

3. Results and discussion

The physicochemical characteristics of centrifuge effluent water are shown in Table 2. The average pH of this water was neutral (≈7). Kearns et al. (2019) also observed neutral pH values in centrifuge effluent water from a fresh-cut lettuce washing line. The centrifugation process separates water that is in intimate contact with the produce tissue and tissue exudate from the cut edges. Therefore, there is a high concentration of organic matter in the centrifuge effluent water. Accordingly, conductivity, turbidity, and COD of the centrifuge effluent water showed high levels compared with values previously reported for leafy vegetables process wash water (Selma et al., 2008; Van Haute et al., 2015). Furthermore, the concentration of free chlorine in centrifuge effluent samples was always below 5 mg/L.

Table 3 shows the contingency table for the comparison of the presence of *E. coli* in centrifuge effluent water and fresh-cut lettuce. The percentage of positive samples was similar in centrifuge water 17.9% (17 out of 95) and lettuce (22.1%, 21 out of 95). Castro-Ibáñez et al. (2016) did not find *E. coli* in baby spinach (n = 54, limit of detection 0.7 log cfu/g), but they found positive samples of centrifuge water for *E. coli* (n = 26). Previous studies have reported a prevalence of *E. coli* in fresh-cut salads of about 10–20% (Abadias et al., 2008; Valentin-Bon et al., 2008). In our study, the average concentration of *E. coli* in positive water samples was 7.3 cfu/100 mL (0.86 log cfu/100 mL) and the maximum concentration detected was 28 cfu/100 mL (1.4 log cfu/100 mL). Castro-Ibáñez et al. (2016) detected levels of *E. coli* in centrifuge effluent water ranging between 0.2 and 0.8 log cfu/100 mL in a baby spinach processing line. In the present study, the average concentration of *E. coli* in positive fresh-cut lettuce samples was 1.7 cfu/g (0.23 log cfu/g), and the maximum concentration detected was 12.8 cfu/g (1.1 log cfu/g). Valentin-Bon et al. (2008) detected a concentration of *E. coli* ranging between 3 and 9 most probable number (MPN) per gram in spinach and lettuce mixes. In the study by Holvoet et al. (2012), performed in companies in which no disinfectant was used to maintain the microbiological quality of the process wash water, the levels of *E. coli* detected in the end product (fresh-cut leafy vegetables) ranged between < 1 and 2.7 log cfu/g.

Table 2
Physicochemical characteristics of centrifuge effluent water.

Parameter	Maximum	Minimum	Mean ± St. Deviation
pH	7.3	5.9	6.9 ± 0.4
Conductivity (µS/cm)	1997	693	1239 ± 256
Turbidity (NTU)	155	87	108 ± 16
COD (mg O_2/L)	3620	2840	3261 ± 219

Samples (n = 95).

Table 3
Contingency table with the number of positive and negative results for *E. coli* presence in centrifuge water and fresh-cut lettuce. TP: True Positive; FP: False Positive; FN: False Negative; TN: True Negative.

		Fresh-cut lettuce		
		Positive	Negative	Total
Centrifuge effluent water	Positive	TP: 10	FP: 7	**17**
	Negative	FN: 11	TN: 67	**78**
	Total	**21**	**74**	**95**

Contingency tables are one of the main ways of evaluating the performance parameters in qualitative analysis, and they are used to assess the overall performance of screening assays (Pulido et al., 2003). In our study, the rate of false positives was 9.5%, as linked to the 74 samples negative for *E. coli* in lettuce, there were 7 positive centrifuge water samples (Table 3). In this case, the presence of 'false positives' could indicate instances in which the analysis of centrifuge water was able to detect the presence of *E. coli* when it was not possible by analyzing the produce due to the well-known heterogeneity of the contamination in fresh produce. On the other hand, the rate of false negatives was 52.4%, as linked to the 21 samples positive for *E. coli* in lettuce, there were 11 negative centrifuge water samples. The validity of an alternative test must be assessed using estimates of sensitivity, specificity and efficiency (AOAC, 2006). The sensitivity of the alternative method was 47.6% (10 positive water samples linked with 21 positive lettuce samples), specificity was 90.5% (67 negative water samples connected with 74 negative lettuce samples), and efficiency was 81.1% (77 coincident results out of 95 matchings). The analysis of centrifuge water to detect the presence of *E. coli* in fresh-cut lettuce showed high specificity and efficiency values. However, the reliability of this alternative method would be low due to its low sensitivity associated with a high percentage of false negative results.

The outcome of the present study regarding the detection of *E. coli* in centrifuge effluent water is in contrast with the results of different studies performed at lab- and commercial-scale with produce inoculated with the target microorganisms (Buchholz et al., 2012; Tomás-Callejas et al., 2012; Castro-Ibáñez et al., 2016; Kearns et al., 2019). It is known that the behavior of inoculated microorganisms is not necessarily similar to that of the natural microbiota of produce. Gomez et al. (2010), for example, observed that the recovery of inoculated bacteria in centrifugation water is higher than that of the natural microbiota. It is important to emphasize that in the present study, un-inoculated commercial-scale samples were analyzed which represent real situations.

Additionally, the chosen methodology might have an impact on the obtained results. In this study, water centrifugation was used to concentrate *E. coli* present in the centrifuge effluent. As specified in Sobsey, 1999, initial concentration and recovery of microorganisms, including bacteria and parasites, are sometimes done by sedimenting the cells using centrifugation. Typically, bacteria and parasites can be sedimented from water and other aqueous samples at relative centrifugal forces of several thousand times gravity for several minutes. Once the supernatant water is removed, the sedimented cells can be resuspended in a small volume of water for subsequent analysis and characterization. This is necessary because of the nature of the centrifuge effluent, which shows very high organic matter concentration and turbidity, which difficulties the filtration of the samples. This is a widely accepted

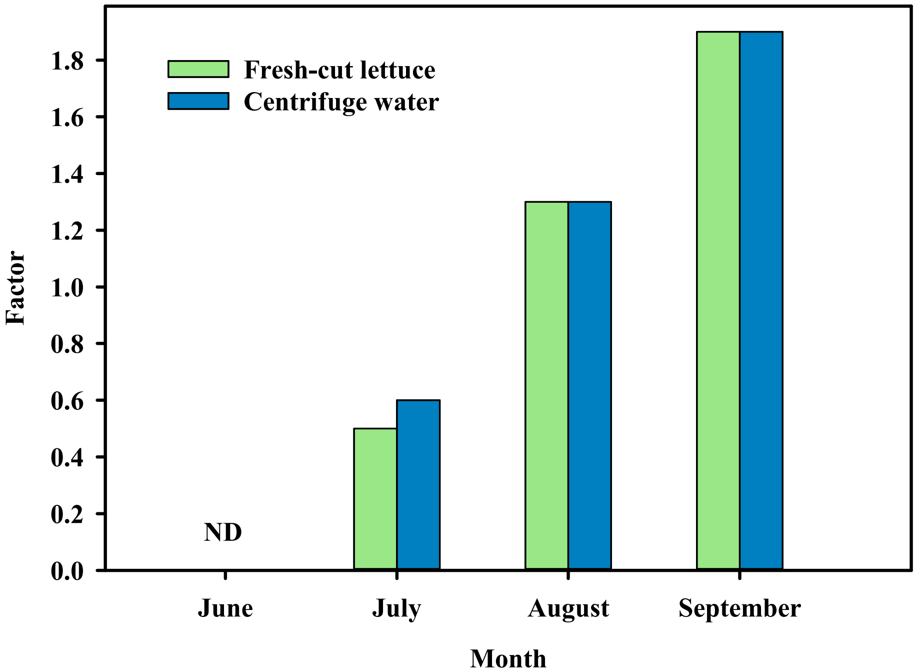

Fig. 1. Variability in *E. coli* presence in fresh-cut lettuce and centrifuge effluent water based on the sampling month. The factor was calculated as the percentage of the total number of positive samples in a specific month divided by the percentage of the total number of samples taken the same specific month. ND: No positive samples detected.

solution, but other strategies could have led to different results reducing the false negatives while increasing the sensitivity. This is the case of using molecular-based techniques combined with the use of dyes (Truchado et al., 2016).

The proportion of positive samples in centrifuge water and finished product varied similarly over the different sampling months (Fig. 1). There were no differences in the percentage of the total positive water samples divided by the total water samples at each month respect to results on the finished product. The higher proportion of positives for *E. coli* in August and September compared with June and July could be linked to the weather conditions of the produce growing fields in the different months that affect the preharvest survival of microorganisms (Castro-Ibáñez et al., 2016). Ailes et al. (2008) detected higher counts of index microorganisms in the fall (September, October, and November) when compared to spring and winter when analyzing cilantro and parsley in the southern USA. On the other hand, Allende et al. (2017) detected a higher prevalence and concentration of *E. coli* in baby spinach in winter compared to spring.

When the results of centrifuge water were examined at different time slots over the same sampling day versus the finished sample, the same differences between time slots were observed in water and lettuce (data not shown). The variations detected between different time slots during the sampling period could have been caused by the dissimilarities in the production rate that is rather variable in most processing lines. The two time slots that showed the lower presence of *E. coli* (9:00–10:00 h, and 13:00–13:30 h) corresponded to the time before meal breaks when the amount of produce in the line was probably smaller compared with other sampling times. When the ratio produce/wash water is low, there is lower disinfectant demand in the washing tanks and, as a consequence, the water and produce disinfection is expected to be more efficient, leading to the lower prevalence of *E. coli* in fresh-cut produce and centrifuge effluent water. On the contrary, if the production rate is very high, the disinfectant demand is very high, and the number of positive samples in the finished product can be higher. Unfortunately, data on the amount of produce processed in the different time slots were not available, and this hypothesis could not be confirmed.

In the present study, *E. coli* was used as an index microorganism of the potential presence of pathogens (i.e., food safety) due to the low prevalence of pathogenic microorganisms in fresh-cut produce environment. Higher levels of index organisms may (in certain circumstances), correlate with a higher probability of an enteric pathogen (s) being present (Ceuppens et al., 2015). However, it has to be taken into account that the reliability of microbial index to predict the presence of pathogenic microorganisms might be questionable depending on the situation (Ceuppens et al., 2015; Allende et al., 2018).

4. Conclusions

Both matrices, centrifuge effluent water, and fresh-cut lettuce showed a similar proportion of positive results for the presence of *E. coli* (≈20%). Furthermore, in most of the cases (81%), the result in lettuce and centrifuge water was coincident. However, about half of the samples positive for *E. coli* in lettuce did not match with water samples positive for *E. coli*. According to the results of the present study, although the analysis of centrifuge water would be a method with high specificity and efficiency to detect the presence of *E. coli* in fresh-cut lettuce, it would not be a reliable alternative due to its low sensitivity. However, further studies including larger datasets and alternative detection methods (e.g., larger volumes of water, use of molecular methods) should be performed in order to confirm or reject the conclusions of the present work.

Declarations of interest

None.

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