Microplastics in Lettuce: The Impacts of Packaging on Dietary Microplastic Exposure Through Produce

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Abstract

Microplastic contamination has become so ubiquitous as to pose a significant risk to the spheres of ecosystem and human health across the world. Dietary microplastic contamination has been of significant concern recently as a potential pathway for bioaccumulation of microplastics in humans. However, this body of literature has developed a bias towards the risk of contamination through seafood, as microplastic contamination in the ocean is particularly high. This research investigated microplastic levels in romaine lettuce intended for human consumption and investigate the role of plastic packaging in potential microplastic contamination in order to gain a new perspective on dietary exposure routes. In comparing packaged and unpackaged lettuce, no difference between the two groups was detected (p-value = 0.5839). This inspires further questions concerning the extent of laboratory microplastic contamination and the future of research into microplastics and produce.

1. Introduction

Microplastics, small fragments of plastics often undetectable to the naked eye, have become a consistent component of the environment of the entire biosphere. Plastics and their additives have been located throughout every major body of water and in many species, including humans (GESAMP 2016). The ubiquity of microplastics is of particular concern as many plastic additives, such as BPA, have been shown to be harmful to human health (Calafat et al. 2008), which has led to a growing concern around dietary microplastic exposure. One particular area of consumption which has not had significant scientific investigation is that of fresh produce. To address this gap, we evaluated romaine lettuce, a previously unresearched food item, and the impact which plastic packaging may have on its observed microplastic levels.

To date, no significant scientific investigation into produce has been conducted. Due to the density of microplastics in the ocean, investigation into microplastic proliferation has largely concerned oceanic products, such as seafood (Karbalaei et al. 2018). Additionally, several studies focus on these products well before they are intended for human consumption, including organs which usually are not consumed by humans, such as the gastrointestinal tract (Baalkhuyur et al. 2018, Rochman et al. 2015, Karlsson et al. 2017). The significant research conducted on non-marine dietary microplastics was largely focused on beverages and solubles, such as honey, salt, and beer (Karami et al. 2017, Iñiguez et al., Liebezeit and Liebezeit 2013, 2014). While microplastics were found in all of these, contamination was similarly pervasive (Lachenmeier et al. 2015). Among all of this research, no assessment of microplastics in fresh produce was conducted. By investigating an under-researched product, produce, at the stage of production nearest to consumption (in the grocery store), this research will create a more robust understanding of how all foodways impact human microplastic exposure, and how processing tactics, such as packaging, play a role in exposure.

For the purpose of this particular research, the overarching questions to investigate are: Are there detectable microplastics in romaine lettuce, a very common food item, intended for human consumption? If yes, do microplastic concentrations vary depending on whether or not they were packaged in plastics? Because of romaine's availability both in unpackaged heads and prepared plastic bags, it serves as the ideal produce through which to investigate how the phenomenon of how packaging impacts microplastic exposure. Using romaine lettuce, this research begins an investigation into the role of both produce and packaging in microplastic exposure.

2. Methods

2.1 Sample Preparation

This protocol is adapted from the methods of Loder et al. 2017, Maes et al. 2017, Wang and Wang 2018, Karlsson et al. 2017, and Quinn et al. 2018. Two unpackaged heads of romaine lettuce and two heads of romaine lettuce in plastic packaging were purchased from 5 nearby stores - Sprouts, Stater Bros., Cardenas, El Super, and Super King. Upon purchasing the lettuce, it was carried out in sealed individual paper bags to avoid contamination. In the lab, unbagged lettuce was thoroughly washed with deionized water, while bagged lettuce leaves were not washed, as per the recommended health guidelines for preparing unwashed and pre-washed lettuce (Palumbo et al. 2007). Lettuce leaves were inserted into a Hamilton Beach Glass Jar Blender Black (Model Number 54216) until it was filled to the top. 200 mL of Milli-Q deionized water were added to the blender. The solution was blended for 30 seconds on the slower "grind" setting (approximately 3,100 rpm), and then for 60 seconds on the faster "cream" setting (approximately 3,700 rpm). The contents of each beaker were strained through a stainless-steel filter with a mesh size of 5 mm to capture larger organic fibers while allowing all particles meeting the qualification of "microplastic" (<5mm) to pass through until 100 mL of solution was obtained. This solution was placed in a glass beaker covered with aluminum foil to avoid airborne contamination. 5 mL of the blended lettuce solution was extracted using a glass pipette and placed in a 100 mL glass beaker. Due to the large quantities of cellulose in lettuce, the enzyme cellulase from *Aspergillus niger* (purchased from Fisher Scientific) was used to digest the remaining organic material while leaving any microplastic particles intact. 5 mL of cellulase and 10 mL of a phosphate-buffered saline (PBS) solution (1 L prepared with 8 g NaCl, 200 mg KCl, 1.44 g Na_2HPO_4 and 240 mg KH_2PO_4 in deionized water, set to pH 5.0 using hydrochloric acid) were added. The beaker was covered with aluminum foil, and the solution was incubated at 50 degrees C for 4 days. These steps were repeated for every purchased head of lettuce.

When the incubation period was completed, NaCl in solution with deionized water (density=1.2g/mL, stirred for 10 minutes) was added to the incubated solution until the beaker had 100 mL of solution in it. The solution was left to settle for 30 minutes, then a vacuum system was used to collect the top 40 mL of each sample and any floating microplastics within it. 5 mL of 0.08 g/mL Nile red dye solution was added to this extracted solution and allowed to stain for 30 minutes. This extracted and stained solution was run through vacuum filtration with an entirely glass apparatus to separate stained fibers from their liquid matrix. The resulting filter paper was placed in a glass beaker, covered with aluminum foil, and allowed to dry overnight. R was used to generate random coordinate points. A grid was created on the dried filter paper, and four random points were plotted onto this grid on each paper. The digital Revolve microscope with fluorescent light (4x zoom lens, TXRED fluorescent/FL overlay at 100% brightness, ~110 ms capture) and ocular techniques were used to count the number of fluorescent particles present at each marked location on the paper. These steps were repeated with each incubated solution.

To mitigate the impacts of contamination or procedural error, the results were corroborated through the use of blanks. 3 water "samples" went through this protocol alongside the experimental samples, but with 100 mL of deionized water rather than solution material. All equipment used was washed thoroughly before and after touching any sample, and all water used to wash was deionized so as to avoid contamination from the water.

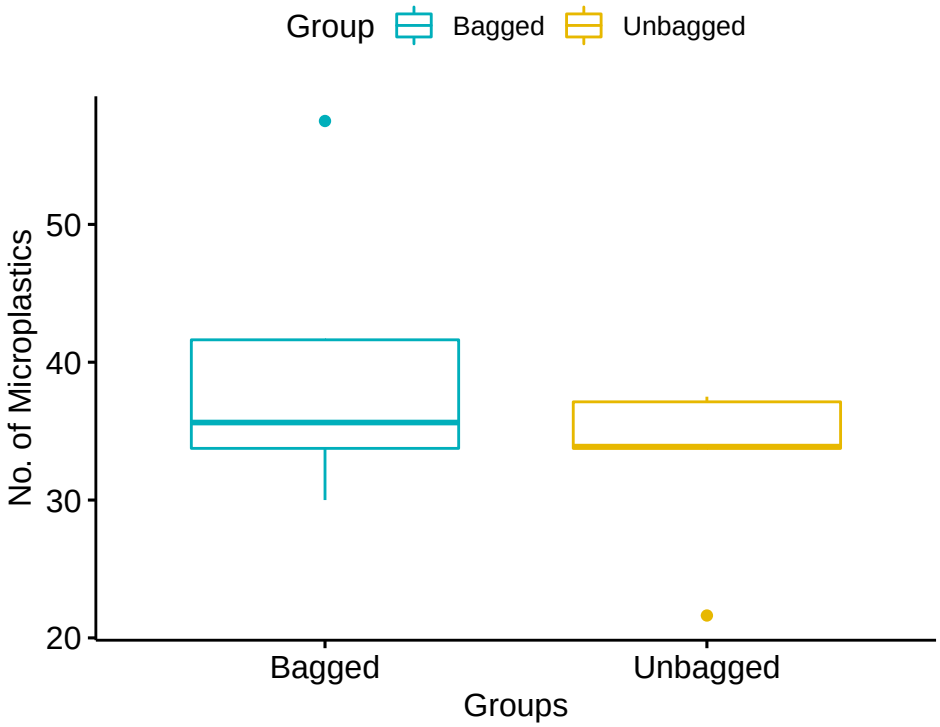
2.2 Statistical Analysis

The data collection yielded five replicates and five pseudo-replicates for both bagged and unbagged lettuce, as two bagged and unbagged heads were purchased at five different stores. In order to more accurately reflect these replicates, the number of microplastics found on each head of lettuce was averaged with its pair. For example, the number of microplastics found on each bagged lettuce head purchased at Cardenas were averaged with one another. This experiment was designed to test the null hypothesis of "there is no difference in microplastic quantities in romaine lettuce with or without plastic packaging." In order to determine whether or not to reject the null hypothesis, whether or not the data fell within normal distribution was first assessed, using Q-Q Plots and the Shapiro-Wilk normality test (See Appendix II). The data for the

unbagged lettuce was found to diverge from the normal distribution, so a Paired Samples Wilcoxon Test was used to assess whether the difference between the bagged and unbagged lettuce were statistically significant.

3. Results

The averages showed that bagged lettuce generally had slightly more microplastics than unbagged lettuce, as is shown in the graph below. However, the Paired Samples Wilcoxon Test yielded a p-value of 0.5839, indicating no statistically significant difference between bagged and unbagged lettuce.



5. Discussion

At least 5, and up to 103, microplastics were observed at each point on each piece of filter paper which was analyzed. As noted in the methods, the filter papers were generated from 5 mL of lettuce, and only four points (each one 400 micrometers across) were observed and averaged from each filter paper. Because the scope of this experiment examined a section of lettuce much smaller than what is typically consumed, it is possible that the quantity of microplastics consumed when eating romaine lettuce. The numbers which were observed in this experiment were not dissimilar from those found in other, similar studies - even those involving notoriously contaminated seafood - as is shown in the table below. As most of these experiments uses grams of either fresh or wet weight to evaluate microplastic concentrations, using fresh weight rather than mL in the future may be more conducive to readily comparing these results with those from different experiments. Even so, based on the relatively high numbers found in this study indicate that microplastics in produce may be an issue of significant concern which should be investigated further, particularly as the exposure levels from seafood have been shown to pose a risk to human health (Barboza et al. 2018).

Author	Matrix	Unit	Minimum	Maximum
Fossi et al. (2015)	Fin Whales	number microplastics/meters cubed	0.0	9.67
Baalkhuyur et al. (2018)	Red Sea Fishes	number microplastics/gastrointestinal tract	0.0	3.00
Rochman et al. (2015)	Fish and Bivalves	number microplastics/gastrointestinal tract	0.0	21.00
Cauwenberghe and Janssen (2014)	Bivalves	number microplastics/grams wet weight	0.0	0.63
Devriese et al. (2015)	Brown Shrimp	number microplastics/grams wet weight	0.0	1.23
Kolandhasamy et al. (2017)	Mussels	number microplastics/grams (by organ)	1.0	10.00
De Witte et al. (2014)	Blue Mussels	number microplastics/10 grams	2.6	5.10
Iñiguez et al. (2017)	Spanish Table Salt	number microplastics/kg	43.0	283.00
Karami et al. (2017)	Commercial Salts	number microplastics/kg	0.0	10.00
Liebezeit and Liebezeit (2013)	Honey and Sugar	number microplastic fibers/500g	20.0	330.00
Liebezeit and Liebezeit (2014)	German Beer	number microplastics/L	16.0	254.00

That being said, there was a significant risk of contamination in this experiment. As was noted by Lachenmeier et al. (2015), methodologies for finding microplastics have consistently struggled to avoid contamination, as air and water oftentimes contains microplastic particles. Although great care was taken to ensure that samples were covered at all times, and only deionized Milli-Q water was used in association with this experiment, laboratory contamination was not avoided in this experiment either (Wang and Wang 2018). It is possible that the deionized water had some remaining microplastics in it. Further methods development Although water is a very necessary element of experiments such as these, future experiments may consider testing the deionized water itself for microplastics before conducting the experiment. Another major contamination concern is airborne contamination (Lachenmeier et al. 2015). Some further precautions may be put in place to reduce plastic concentrations in the laboratory. A reduction of the plastics present in the lab prior to the conduction of this experiment, closing the air vents prior to and for the duration of the experiment, an investment in cotton lab coats and gloves (polyester and latex were used to complete this experiment), and perhaps the completion of the entire experiment under a laminar flow hood may help to reduce airborne microplastic contamination (Wesch et al. 2017, Woodall et al. 2015). Additionally, as airborne contamination is a large concern, testing the air in the laboratory for microplastics may help to provide some context as to where the contamination is coming from, and perhaps indicate more concretely how that contamination may be minimized (Prata 2018, Wesch et al. 2017).

Throughout the literature, the most common method used to break down biotic material is acidic and basic digestion. Cauwenberghe and Janssen (2014), Baalkhuyur et al. (2018), Kolandhasamy et al. (2018), Rochman et al. (2015), Devriese et al. (2015), and de Witte et al. (2014) all used an acid or a base to digest the organic material and prevent the false identification of organic fibers and synthetic. However, the use of enzymatic digestion (as opposed to relying on corrosive acids or bases) can be beneficial in preventing

false negatives and subsequent underestimation, as corrosive compounds can break down microplastics or render their chemical composition unidentifiable (Karlsson et al. 2017, Loder et al. 2017. While acids and bases have the potential to break down both plastics and biotic material, enzymes are often equally effective in eliminating biotic material while preserving the integrity of the microplastics themselves (Loder et al. 2017). Because of the concern around the loss of microplastic particles when using a corrosive acid or base digestion, enzymatic digestion was used to complete this isolation of microplastics. However, this led to some methodological issues may have contributed to potential error in the data obtained. One particular issue which was encountered was that, when straining the blended lettuce solution in order to create a solution liquid enough to digest, the fibrous remains largely accumulated on top of the sieve and did not filter into the solution used to produce the filter papers which were observed, which may have led to the underestimation of how many observed plastics were in the lettuce, as many particles may have become trapped in this pile and not made it into the experimental solution. Another issue which was encountered was the difficulty to determine which particles viewed under the microscope were synthetic, and which were simply undigested lettuce fibers. A misinterpretation of remaining biotic material as plastics may have led to an overestimation of the microplastics present in the samples. One way which both of these issues could be resolved is through a more thorough enzyme digestion, similar to the one described by Loder et al. (2017). When completing a full enzymatic digestion, the blended lettuce would not have to be strained, as the approximately three week-long process would thoroughly break down the biomatter on its own. In order to improve the success of the enzyme digestion, adding more water than 200 mL may be beneficial, as the enzyme protocol was designed for and most effective with seawater containing biota. Additionally, the longer digestion process would make it less likely that biotic fibers would remain in the solution and perhaps be mistaken as microplastic particles. If the time constraint is too significant to complete a full digestion process, a combination of boiling and nitric acid could also be used to eliminate biotic material, as per the methods of Rochman et al. (2015), albeit risking potential damage to and subsequent underestimation of microplastics in the material.

One additional improvement which may be made to similar research in the future may be found in the number of replicates used. On the box plot, there appears to be a slight trend towards more microplastics being present on bagged rather than unbagged lettuce, however, with the number of replicates which were used in this research, that trend is not statistically significant. Additionally, the average of the blanks, when subtracted from the averages of each experimental replicate, yielded positive values for all but three experimental samples. This indicates that there likely are plastics above the background level in the majority of these samples (85%). However, the power to detect difference between the two types of lettuce in this experiment was low (Beta = 0.41). To avoid a Type II error, this experiment would need to be repeated with 26 samples from each experimental group would be needed, plus blanks.

Due to the magnitude of work necessary to reproduce these results with more replicates and more thorough enzymatic digestion, this particular experiment likely should not be repeated without a much longer time frame available for its completion. If this experiment could be re-conducted over the course of at least two months, or even a semester, then it would be interesting to investigate whether or improving the enzymatic digestion process or increasing the number of replicates produced statistical significance. Additionally, many past articles have noted the need for further method development in preventing microplastic contamination (Karlsson et al. 2017, Lachenmeier et al. 2015). As this experiment observed microplastic quantities similar to those observed in other, more researched dietary arenas, further research into whether or not these values are of concern, or likely due to contamination, are warranted. Experiments comparing microplastics found on various produce with background levels found in laboratory air or water samples may be useful in concluding whether or not microplastics in produce are a genuine concern (Lachenmeier et al. 2015, Prata 2018, Wang and Wang 2018, Wesch et al. 2018). This reality underscores the necessity, discussed by Rist et al. (2018), of expanding and contextualizing the debate around microplastics. The microplastics observed in lettuce samples, as well as the high levels of laboratory contamination, indicates how universal microplastics are in almost all environments today. As this report indicates, areas which were previously considered to be unlikely sources of microplastic exposure may be found to have substantial contamination, underscoring the need for further research into the scope of microplastic exposure (Karbalaei et al. 2018, Rist et al. 2018). Although the high microplastic levels observed may be due to contamination, further research is necessary to affirmatively answer this question.

6. Conclusion

As statistical significance was not obtained in this experiment, it is difficult to speak conclusively as to what may be learned from this research. In many ways, this experiment simply provides a foundation on which more targeted questions may be built. However, the relatively high levels of microplastics found in this experiment indicate that produce microplastic exposure may be comparable to areas more traditionally considered to be high-risk, such as seafood. This reality underscores the necessity, discussed by Rist et al. (2018), of expanding and contextualizing the debate around microplastics. Microplastics have become entirely omnipresent across essentially all global environments, so much so that no laboratory protocol has been designed which may entirely eliminate the risk of microplastic contamination. While it is of course important to understand the risks posed by a threat as universal as microplastic exposure, dietary pathways are still just one of many routes through which individuals are exposed to microplastic pollution every day. While this indication that microplastics may be present in produce as well as other more traditionally high-risk foods is certainly unnerving, it remains just one piece in a large web of constant microplastic exposure. The microplastics observed in lettuce samples, as well as the high risk of laboratory contamination, indicates how universal microplastics are in almost all environments today. As this report indicates, areas which were previously considered to be unlikely sources of microplastic exposure may be found to have substantial contamination, underscoring the need for further research into the scope of microplastic exposure.

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Appendix I: Raw Data

X	Store	U.B....Bagged	Sample..	Point.1	Point.2	Point.3	Point.4	Average
Cardenas U.B. #1	Cardenas	UB	1	5	10	12	13	10.00
Cardenas U.B. #2	Cardenas	UB	2	32	52	38	11	33.25
Cardenas B. #1	Cardenas	BG	1	70	62	84	103	79.75
Cardenas B. #2	Cardenas	BG	2	37	32	30	42	35.25
El Super U.B. #1	El Super	UB	1	76	29	24	18	36.75
El Super U.B. #2	El Super	UB	2	38	44	32	36	37.50
El Super B. #1	El Super	BG	1	37	31	36	33	34.25
El Super B. #2	El Super	BG	2	53	52	52	39	49.00
Sprouts U.B. #1	Sprouts	UB	1	31	34	19	32	29.00
Sprouts U.B. #2	Sprouts	UB	2	54	20	66	15	38.75
Sprouts B. #1	Sprouts	BG	1	36	45	51	52	46.00
Sprouts B. #2	Sprouts	BG	2	28	19	20	34	25.25
Stater Bros U.B. #1	Stater Bros	UB	1	33	45	37	51	41.50
Stater Bros U.B. #2	Stater Bros	UB	2	47	24	34	29	33.50
Stater Bros B. #1	Stater Bros	BG	1	21	58	28	20	31.75
Stater Bros B. #2	Stater Bros	BG	2	28	22	36	27	28.25
Super King U.B. #1	Super King	UB	1	10	26	23	34	23.25
Super King U.B. #2	Super King	UB	2	57	21	33	66	44.25
Super King B. #1	Super King	BG	1	22	24	26	27	24.75
Super King B. #2	Super King	BG	2	46	41	45	39	42.75
Blank #1	Blank	Blank	1	18	10	55	31	28.50
Blank #2	Blank	Blank	2	28	27	8	12	18.75
Blank #3	Blank	Blank	3	24	46	25	13	27.00

Appendix II: Normality Tests

In order to determine whether or not to reject the null hypothesis, whether or not the data fell within normal distribution was first assessed, using Q-Q Plots and the Shapiro-Wilk normality test.

For bagged lettuce data, the Shapiro-Wilk normality test yielded a p-value of 0.2768, indicating that it is within the bounds of normal distribution. The Q-Q plot, shown below on the left, also demonstrates that most points fall within the expected bounds.For unbagged lettuce data, the Shapiro-Wilk normality test yielded a p-value of 0.04526, indicating that it is not within the bounds of normal distribution. The Q-Q plot, shown below on the right, also demonstrates that, while most points fall within the expected bounds, some are much further out of normal distribution than they were for the bagged lettuce data.