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Determination of the potential bioavailability of plant microRNAs using a simulated human digestion process

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Abbreviations: cDNA, complementary DNA**; cel-lin-4**, *Caenorhabditis elegans*-lin-4 miRNA, **Cq,** quantitation cycle; **LEA**, Late embryogenesis abundant; **mRNA,** messenger RNA; **miRNA**, microRNA; **qRT-PCR**, quantitative real-time PCR; **RNase**, Ribonuclease; **RQI**, RNA Quality Index; **SGF**, simulated gastric fluid; **USP**, United States Pharmacopoeia

Key Words: Cross-kingdom regulation, Diet, MicroRNA, Nutraceutical, Simulated digestion

Abstract

Scope

The “dietary xenomiR hypothesis” proposes that microRNAs (miRNAs) in foodstuffs survive transit through the mammalian gastrointestinal tract and pass into cells intact to affect gene regulation. However, debate continues as to whether dietary intake poses a feasible route for such exogenous gene regulators. Understanding on miRNA levels during pre-treatments of human diet is essential to test their bioavailability during digestion. This study makes the novel first use of an *in vitro* method to eliminate the inherent complexities and variability of *in vivo* approaches used to test this hypothesis.

Methods and results

Plant miRNA levels in soybean and rice were measured during storage, processing, cooking, and early digestion using real-time PCR. We have demonstrated for the first time that storage, processing, and cooking does not abolish the plant miRNAs present in the foodstuffs. In addition, utilizing a simulated human digestion system revealed significant plant miRNA bioavailability after early stage digestion for 75 min. Attenuation of plant messenger RNA and synthetic miRNA was observed under these conditions.

Conclusion

Even after an extensive pretreatment, plant-derived miRNA, delivered by typical dietary ingestion, has a robustness that could make them bioavailable for uptake during early digestion. The potential benefit of these regulatory molecules in pharma-nutrition could be explored further.

1. Introduction

Small non-coding RNAs are recognized as key components in the post-transcriptional regulation of gene expression in animals and plants [1, 2]. Following the association of miRNAs with their target messenger RNA (mRNA), miRNAs can regulate the expression through mRNA cleavage or translational repression [1, 3]. A single miRNA is able to target over hundreds of transcripts in humans and miRNAs are estimated to regulate over 60% of the total protein coding genes [4].

The innate capacity of miRNAs to resist degradation provides them with their ability to function in their critical role as extracellular regulators but to also survive in unfavorable physiological conditions such as in the presence of extremes of pH (e.g. transit through the gastrointestinal tract) and high ribonuclease (RNase) activity [5]. It is this robustness of miRNA that has brought about the dietary xenomiR hypothesis [6]. Here it is proposed that diet-derived miRNAs (xenomiRs) may survive the digestive process to become part of an animal's circulating miRNA profile, which could go onto regulate the animal's gene function [6]. Support for this hypothesis came in 2012, when a report found plant miRNAs circulating in mammalian blood and evidence of cross-kingdom regulation of a mouse protein by rice miR168a [7]. However, the xenomir hypothesis remains controversial [8, 9]. As human diets are extremely diverse, there may be value in ascertaining which food sources may be more beneficial to human health due to their particular miRNA transcriptome. Recently, Baier *et al.* [10] observed that endogenous miRNA synthesis cannot compensate for dietary deficit and that a regular dietary miRNA intake may be a key to check aberrant gene function.

To date, published reports use animal models or human volunteers to detect exogenous miRNAs in serum to test their survivability in the digestive system and their potential for cross-kingdom gene regulation [7, 8,11]. Such *in vivo* studies face significant challenges in overcoming extra levels of complexity and variables, which may impact on achieving good experimental design [10]. To avoid these complications, we conducted a series of studies on plant miRNAs from highly consumed crops such as soybean and rice using a more defined experimental approach. The levels of plant miRNAs were determined before their digestion i.e. from raw, processed and cooked plant materials. In addition, to examine what happens to the miRNA profiles while they are in a digestive system, we utilized a more unbiased and controlled process of investigation by using a simulated digestion system. This is the first report of an *in vitro* study on dietary miRNA to reveal insight on miRNA survivability both prior to and during digestion.

2. Materials and methods

2.1 Processing and cooking of soybean and rice

Pre-packed EU-originating dried raw soybean (*Glycine max*) and brown rice (*Oryza sativa*) of regular consumer standard was stored at room temperature until used. A modified protocol with an extended boiling duration based on a standard cooking method developed by the National Soybean Research Laboratory was followed to cook soybean [12]. For this study, the cooking time of soybean was extended to 80 mins in order to get a softer cooked bean which might be more acceptable to an average human consumer. A typical adult intake of soybean or rice per serving was used for the experiments. Twenty g of soybeans were soaked in 150mL RNase-free

water (Lonza, Slough, UK) with 0.25% (w/v) $NaHCO_3$ [13] overnight at 4°C. The beans were separated from the soaking liquid, rinsed in fresh RNase-free water and then boiled in 600 mL RNase-free water for 80 min until they turned soft in texture. The rice was rinsed in RNase-free water and cooked for 25 min in 300 mL of boiling RNase-free water. The cooked plant material was briefly homogenized with a pestle for 10-20 seconds to replicate mastication prior to *in vitro* digestion. Fifty mg each of the powdered raw beans/rice, cooked beans/rice, 100 µL of the liquid in which the beans were soaked and 100 µL of the water in which the rice/beans were cooked were retained for total RNA extraction.

2.2 In vitro digestion of soybean and rice

In vitro digestion of the homogenized plant material was carried out using a drug dissolution tester TDT-08L (Pharma Alliance Group Inc, California, USA). The drug dissolution tester is an industry standard system complying with United States, International, and European Pharmacopoeia specifications (USP, IP, and EP), which is routinely used to provide *in vitro* drug release information and to predict *in vivo* drug release profiles by simulating the physiological conditions of a human gastrointestinal tract [14,15]. In this study we regarded soybean seeds as tablets capable of releasing nutraceutical components during digestion. The system was used with simulated gastric fluid (SGF) with a pH of 1.2 at a temperature of 37°C and a simulated gut movement of 50 rpm with the help of the attached paddles. The SGF contained 3.2 mg/mL pepsin and 0.03M NaCl at pH1.2 as per the USP standards [16]. The half gastric emptying time ($T_{1/2}$) of a solid test meal is 69 min [17]. Therefore, in order to determine the miRNA profiles during the initial stages of digestion, at time intervals of 15 min, 30 min, 45 min, 60 min, and 75 min of

incubation, individual 100 µL aliquots of samples were collected for total RNA extraction. The whole process of soaking, cooking, *in vitro* digestion, and sampling was carried out in triplicate.

2.3 RNA extraction

The total RNA including small RNAs from the soybean and rice samples such as raw, soaked, cooked, digested at 15 min, 30 min, 45 min, 60 min, 75min, soybean soaked water and soybean/rice cooked water were carried out using a PowerPlant Total RNA isolation kit (Mo Bio, Carlsbad, USA), following the manufacturer's instructions for the combined total RNA and small RNA isolation protocol. The RNA concentrations of all samples were measured spectrophotometrically using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Loughborough, UK).

2.4 Small RNA Quality Analysis

RNA quality, integrity, and quantity assessments which included small RNA recovery was examined by an Experion automated micro-fluidic electrophoresis system (Bio-Rad, Hemel Hempstead, UK) and a StdSens Total RNA assay kit (Bio-Rad, Hemel Hempstead, UK).

2.5 cDNA synthesis of mature miRNAs

The TaqMan miRNA cDNA synthesis kit (Applied Biosystems, Paisley, UK) was used to carry out stem-loop reverse transcription of extracted total RNA for quantitative real-time PCR (qRT-PCR) in order to quantify plant miRNAs using TaqMan miRNA assays (Applied Biosystems, Paisley, UK). TaqMan microRNA assays use a miRNA target-specific stem-loop reverse transcription primer to permit the specific detection of the mature, biologically active miRNA for each assay. The stem-loop primer

extends the 3' end of the target to produce a template that can be used in standard TaqMan assay–based real-time PCR. Total RNA isolated from the plant samples were used as templates for cDNA synthesis. Each reaction was carried out as per manufacturer's instructions and contained 10 ng of total RNA, 50 nM stem–loop RT primer, 1X RT buffer, 0.25 mM each of dNTPs, 50U of MultiScribe reverse transcriptase and 3.8U of RNase inhibitor. The 15 μl reactions were incubated in a Model 480 thermocycler (Perkin-Elmer, Beaconsfield, UK) for 30 min at 16°C followed by 30 min at 42°C, 5 min at 85°C and finally held at 4°C. No template control (NTC) reactions, which contained ultra-pure water instead of total RNA, were also run in parallel.

2.6 Quantitative Real-Time PCR

TaqMan small RNA quantification was performed to assess the survival of plant miRNAs in soybean and rice at various stages of treatments. TaqMan small RNA assays containing primers and probes for quantification were used as follows: gma-miR166a [MI0000201 (000347)], gma-miR167a [MI0000208 (000348)], gma-miR168a [MI0000210 (000351)], cel-lin-4 [MI0000002 (000258)]. Applied Biosystems states that the TaqMan gma-miR166a, gma-miR167a, and gma-miR168a assays will detect these miRNAs in a number of plants species including soybean and rice. The 20 μl reaction mixture contained 1.33 μl stem loop cDNA, 2X Luminaris Color Probe high ROX qPCR master mix (Thermo Scientific, Loughborough, UK) and 1X TaqMan Small RNA assay containing the respective primers and probes. The assay reactions were incubated at 50ºC for 2 min and 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 min in a StepOnePlus RT-PCR system (Applied Biosystems, Paisley, UK) as per manufacturer's instructions.

Real-time PCR quantitation determination utilises the quantitation cycle (Cq) at which the increase in miRNA probe fluorescence is exponential [18]. The TaqMan Cq values were converted into absolute copy numbers using a standard curve constructed from serial dilution of synthetic cel-lin-4 miRNA (Eurofins MWG Operon, Ebersberg, Germany) [19]. In addition, lin-4 was also used as a negative control where the lin-4 assay was used to highlight any non-specific amplification in the total RNA from processed plant samples.

2.7 Analysis of mRNA integrity

The mRNA levels in the soybean seeds at various stages of treatment were assessed using the soybean GMpm16 transcript coding for LEA protein [20]. The total RNA from various samples was used as a template for cDNA synthesis and the reaction was carried out using a Tetro cDNA synthesis kit (Bioline, London, UK) as per the manufacturer's instructions with each reaction containing 100 ng of total RNA, 1 μL oligo(dT), 1X RT buffer, 0.5 mM of dNTPs, 200U of Tetro reverse transcriptase and 10U of RNase inhibitor. The 20 μL reactions were incubated in a Model 480 thermocycler (Perkin-Elmer, Beaconsfield, UK) for 30 min at 45°C. The real-time PCR was carried out using 1X PrimeTimeStd hydrolysis probe qPCR assay (Integrated DNA Technologies, Leuven, Belgium) and 2x Luminaris Color Probe High ROX qPCR master mix (Thermo Scientific, Loughborough, UK) and 1.33 μL cDNA in a reaction volume of 20 μL. The real-time PCR reactions were carried out as described in Section 2.6.

2.8 Simulated food processing, cooking, and artificial digestion of synthetic miRNA

Soybean and rice miRNAs are present within the cellular matrix of the plant material and may be associated with other plant molecules such as proteins and polysaccharides. These plant components along with some possible post-translational miRNA modifications, may provide plant-derived miRNAs with some degree of protection from degradation [21].To see how miRNA free from such complexes or modifications might survive our simulated conditions of food processing, cooking, and artificial digestion, we used synthesised cel-lin-4 miRNA to be assured of a complete absence of any plant products, endogenous RNases, or modifications. To replicate the soaking of the soybeans, 700,000 copies of synthetic cel-lin-4 miRNA in solution was used as the starting material as it showed an equivalent Cq value as that obtained for raw soybean miR166 and was incubated in RNase-free microcentrifuge tubes with 0.25% (v/v) $NaHCO_3$ in a final volume of 50 µL overnight at 4°C. The lin-4 sample containing tubes were then incubated in boiling water for 80 min to simulate the cooking process of soybean. The boiled lin-4 samples (50 µL) were suspended in 531.9 µL, of SGF (the precise volume to replicate the ratio of soybeans to SGF) and incubated at 37°C at 50 rpm in a MaxQ 4000 orbital shaker incubator (Thermo Scientific, Loughborough, UK) for 15 min, 45 min, and 75 min. The samples at each stage were collected for RNA extraction and quantification as specified earlier.

2.9 Heat and RNase treatments on the synthetic cel-lin-4

Heat and RNase treatments were conducted in order to analyze the capability of synthetic miRNA to withstand physical and chemical stress, respectively. In addition, the specificity of the miRNA assays for the precise detection of intact molecules from samples which are degraded at varied levels was confirmed. For heat treatment,

700,000 copies of lin-4 in an RNase-free microcentrifuge tube were incubated in boiling water for 80 min, cooled down at room temperature, and then spun down prior to cDNA synthesis and qRT-PCR. For RNase treatment, 700,000 copies of lin-4 miRNA were incubated with 10U of RNase I_f (New England Biolabs, Hitchin, UK) in 1X buffer in a total reaction volume of 10 µL at 37°C in a water bath for 20 min. RNase I_f will degrade single-stranded and double-stranded RNAs. After the reaction the enzyme was inactivated by incubating at 70°C for 20 min. All the samples underwent the RNA extraction process and the subsequent purified RNA was used for qRT-PCR.

2.10 Statistical Analysis. All numerical quantifications represent mean ± standard error of the mean (n, number of independent experimental repetitions). Comparison of multiple samples was performed by one-way ANOVA followed by Tukey's HSD post-hoc tests to calculate p values. Values of $p<0.05$ were considered significant.

3. Results and Discussion

Researchers have observed a possible miRNA cross-kingdom regulation by analysing the serum from model animals fed with plant materials [7, 8, 11]. Even though controlled feeding of experimental subjects was conducted, as there are sequence similarities between animal small RNAs and plant miRNAs, the assays may have specificity issues and thereby, may have compromised the results [9]. The usage of different food sources for analysing a single plant miRNA, miR156, with different experimental subjects led to contrasting results for Zhang *et al.* [7] and Snow *et al.* [11]. The species of plant or the ripening stage of the plant materials, which the experimental subject ingests during (or before) the experiment, can have a significant impact on the miRNAs detected [11]. Considering these factors, we aimed

to adopt a unique approach to resolve some of the ambiguity surrounding this concept. The novelty of the current study is the emphasis on examining the bioavailability of plant miRNAs both prior to ingestion due to the processing and cooking of the plant materials and also while they are in the gut rather than the more complex, standard methodology of bypassing these stages and examining the miRNA levels further downstream in the serum of experimental subjects typical of *in vivo* studies. In addition, this study aims to help clarify the common uncertainty, pointed out in a recent report [10], as to whether plant-based miRNAs are uniquely protected against degradation during food processing and digestion.

3.1 Storage, processing and cooking of plant materials show substantial yet varied miRNA profiles.

Plant-based diets commonly consumed globally by humans, such as soybean and rice were chosen for this study. Soybean has increasingly become part of the modern diet and advances in soybean crop management has seen global production rise over 350% in the last 40 years (http://www.usda.gov/oce/commodity/wasde/latest.pdf) to meet the demand. We have chosen plant miRNAs, namely miR166, miR167, and miR168 for the simulated digestion study as these are present in abundance in a wide number of plants including soybean and rice [9]. In addition, miR166 and miR167 are amongst the most abundant plant mRNA families reported in mammalian samples after a recent *in silico* analysis of small RNA sequencing data [22]. So far, 573 precursor and 639 mature soybean miRNAs have been classified (miRBase, Release 21) [23] and our preliminary human target prediction analysis of the most abundant of the soybean miRNAs has identified a number of potential target genes in humans (data not

included).

Interestingly in this study, despite possibly lengthy transport and storage chain, from field to consumer, in conditions which might not be favourable for RNA preservation, an integrity assessment of the total RNA isolated from soybean returned a good RNA Quality Index (RQI) of 8 (Figure 1A). After RNA extraction and stem-loop qRT-PCR, miR166, miR167, and miR168 were found in substantially high levels in soybean and rice at the storage, processing, and cooking stages (Figure 1B). qRT-PCR amplification of soybean RNA found the highest level for miR167, whereas miR166 showed slightly lower levels than that of miR167, while miR168 showed the least level (Figure 1B). A significant ($p<0.05$) increase was seen in the level of miRNAs measured when the raw beans were soaked overnight with 0.25% (w/v) $NaHCO_3$ and the level remained without any significant changes in the beans even after boiling for 80 min (Figure 1B). The liquids in which the beans had been soaked and cooked were retained for examining the possible release of miRNAs from the beans into solution. The Cq values for the miRNAs in the water remaining after the beans were cooked were lowest among all the other samples measured revealing a significantly high ($p<0.05$) level of miRNA in the boiled cooking water (Figure 1B). In contrast, the soaking solution used for soaking the beans prior to cooking showed the lowest and an insignificant level of miRNA content (Figure 1B).

Rice miRNA levels were tested as a comparative analysis of Zhang's *in vivo* data [7] in an *in vitro* system. Rice is an important crop worldwide and is consumed by nearly half of the world's population [24]. To date, 713 mature rice miRNAs have been identified (miRBase, Release 21). In rice, of the three miRNAs assayed, miR166 was

found at the highest level, whereas miR167 was slightly lower level than miR166 while miR168 showed the least levels, (Figure 2). The raw rice was cooked directly without any prior treatment and showed a slightly diminished level of miRNA than the uncooked material, whereas the retained cooked water into which the miRNA was possibly released showed high levels comparable to that of raw rice (Figure 2). The cooked water had the highest level of miR167, whereas mi166 was found at higher levels than miR167 in all of the other rice samples (Figure 2). All aspects of food processing from soaking, cooking, and chemical and enzymatic digestion contribute to the weakening and degradation of the food matrix itself in an attempt to break down the food into a state to maximise adsorption by the subject [25]. The chemical treatment ($NaHCO_3$) during soaking and the heat treatment during cooking aid disintegration of cotyledons and thereby facilitate an improved extraction and recovery of these small RNAs [25]. This would be the probable reason for the higher copy number of miRNAs in the soaked and cooked bean samples compared with the raw, uncooked beans and rice. Boiling of the material would have caused disintegration of the cell wall structure of the intact plant material, thereby releasing large amounts of small RNA molecules into the water in which the materials were cooked (Figure.1B and 2).

3.2 Number of miRNA molecules in the simulated gut indicates plant miRNA survivability during digestion.

Different species have anatomical and physiological characteristics that must be considered when utilizing animal models for ingesting plant materials and evaluating their serum for exogenous miRNAs [26-28]. The use of animal models may require complex experimental design which despite of efforts may still yield compromised

results [8, 28]. In order to circumvent such experimental challenges we made use of a drug dissolution tester as a simulated digestion system. Drug dissolution testers are routinely used with highly defined simulated gastric and intestinal fluids by pharmaceutical companies around the world in drug release profiling. These simulated gut systems are designed to provide a strong approximation to the chemical and physical parameters of the human gastric environment to reduce human participation in drug delivery studies [15]. By regarding the plant material in this study as an ingested nutraceutical substance, we made use of the drug dissolution tester in its industrial role to examine the release and stability of plant-derived miRNAs under the physiological conditions of a human stomach.

Even though small 1-3 Cq increases (1.6-10 fold decreases) in measured miRNA levels were observed when cooked soybean and rice was transferred to the digestion system, no significant time-dependent variation in the levels of the assayed miRNAs was observed during the 75 minute incubation in the simulated digestion process (Figures 3A and B). Interestingly, miR168 in soybean and miR166 in rice appeared to show a slightly greater resistance to degradation suggested by their levels unlike the other miRNAs assayed (Figure 3A and B). As there are no sequence differences between the soybean and rice miRNAs examined in this study, this finding might suggest that there may be plant species-specific mechanisms in play, such as their matrix, which may offer varying protection of miRNAs from degradation..

The plant miRNAs copy number per milligram of soybean samples at different stages during the series of treatment was calculated using a cel-lin-4 standard curve in a TaqMan miRNA assay [19]. Confirmation of the specificity of the TaqMan miRNA

assays was obtained using a miRNA assay for cel-lin-4 as a control in soybean RNA samples. As expected, cel-lin-4 copy numbers were negligible compared to the corresponding plant miRNAs (Table.1).

The miRNAs assayed in soybean all followed their rank order as previously determined [9]. miR167 reported the highest copy number in soybean and it maintained its top position throughout the series of treatments while miR166 showed a relatively lower level and miR168 the lowest levels of the miRNAs measured (Table.1). All three plant miRNAs showed the highest copy numbers in the cooked beans, with second highest levels in the soaked beans. The chemical treatment ($NaHCO_3$) during soaking and boiling of the material weakens the cell wall structure of the plant material, which facilitated an improved extraction and recovery of these small RNAs. Cooking of the plant material also releases large amounts of small RNA molecules into the water in which the material was cooked. This may be beneficial where one may wish to process food to reduce the amount of plant-derived miRNAs consumed. Food preparation that encourages a rapid breakdown of the plant material could promote an enhanced release of plant-derived miRNAs during the early stages of digestion. Therefore, drawing parallels with drug release kinetics, the process and timelines of miRNA release and absorption in digestive systems may differ due to the way plant materials are prepared for consumption.

Our *in vitro* digestion study showed consistent miRNA survivability during the early stages of digestion for over an hour. This work lends support to a recent *in silico* study which postulates that plant-derived miRNAs survive degradation in the digestive system in quantities sufficient to make it to the serum and access organs

[22]. With plant-derived miRNAs not as fragile as once thought and a growing interest in the application of miRNA-based genetic modification technology to improve agricultural productivity, the agro-biotech industry may need further research to settle the continuing debate and any possible concerns [29]

Building upon the findings here, future studies could further investigate miRNA survival during the downstream digestion processes, examining the effects of intestinal pH and longer incubation, thereby increasing our understanding of exogenous miRNA survivability right up to any transport into gastrointestinal cells. Such *in vitro* studies, while highly reproducible, are limited in their ability to accurately model the complex biological mechanisms and processes inherent in a human digestive system. However, this groundwork can help build the necessary foundations upon which to pursue, thorough a range of additional experimental approaches, the answer to the crucial question of how dietary miRNAs might be absorbed from the intestine.

3.3 Messenger RNA shows loss of integrity in the processed, cooked, and *in vitro* digested bean samples.

A comparison of the electropherograms of the total RNA extracted from raw (Figure 1A), soaked (Figure 4A), and cooked soybeans (Figure 4B) clearly shows the detrimental effect of food processing on total RNA integrity. To analyse the comparative levels of a messenger RNA during the stages prior to ingestion of the diet, we chose a soybean LEA transcript present in the bean which codes for LEA protein GMpm16 [20]. When mRNA levels are assessed by RT-qPCR, the raw

soybean sample shows the lowest Cq value indicative of the significantly highest LEA mRNA levels among the test samples ($p<0.05$) (Figure 4C). As anticipated with high molecular weight RNA, the subsequent processing and digestion of the soybean samples resulted in a significant and gradual increase in the Cq indicating a gradual decline to a negligible level of detectable mRNA. The soybean samples collected from the digestion system at 15 min time intervals for over an hour showed negligible levels of LEA mRNA. Unlike that of the miRNAs, the solutions in which the soybeans were soaked/cooked showed the highest Cq or the least detectable LEA mRNA in all of the samples measured. To examine the impact of mRNA integrity on the cDNA synthesis of LEA transcripts, oligo(dT) and random hexamers were used to compare their ability to prime the cDNA synthesis of poor quality, degraded mRNA. The raw bean sample showed similar Cq values regardless of the reverse transcription primers used. However, the processed or digested samples showed an average delta Cq decrease of 6.2 when random hexamers were used instead of oligo(dT) indicative of the presence of degraded LEA mRNA transcripts (Data not shown).

The lower susceptibility of plant miRNAs to degradation when compared to that of high molecular weight RNA during food processing and *in vitro* digestion may be due in part to the smaller size of miRNAs, as miRNAs might be less affected by the overall degradation of the total RNA compared to mRNA [30]. Plant miRNA methylation may also make them more resistant to degradation [21, 31].

3.4 Synthetic cel-lin-4 shows higher susceptibility to degradation compared with plant miRNAs and varied level of degradation during heat and RNase treatment.

The observed resilience of plant miRNAs following food processing, cooking, and digestion led to a degradation comparison with synthetic miRNA. The synthesised cel-lin-4 miRNA was free from any possible plant-derived protection to degradation to allow assessment of miRNA susceptibility under these conditions. For these experiments, 700,000 copies of lin-4 miRNA in solution was used as the starting material as it showed an equivalent Cq value as that of the isolated level of miR166 from a standard assay sample of the control raw soybean. The lin-4 synthetic miRNA molecules, which underwent soaking, cooking, and digestion showed the highest Cq values, indicating a significant reduction in levels compared to that of plant miRNAs (Figure 5). This suggests a greater susceptibility of synthetic miRNA to the chemical and physical stresses during simulated food processing, cooking, and digestion than that of plant miRNAs.

Comparison of physical (heat) and chemical treatments (RNase) on synthetic lin-4 was performed and found that the susceptibility of the synthetic miRNA varied according to these treatments. Submitting lin-4 miRNA samples, with a copy number equivalent to that of miR166 in soybean, to 80 minutes of boiling to mimic the soybean cooking process did not completely abolish the molecules and the Cq value of 30 of the boiled sample suggests there are still intact lin-4 synthetic miRNA present after this heat treatment (Figure 6). The synthetic miRNA revealed a sensitivity to heat degradation with a significant fall of 10 Cqs (11,702 fold drop) which was not observed with miR166 measured in soybean after boiling for the same duration. The susceptibility of the synthetic miRNA against RNase was tested by treating with RNase I_f. As RNase I_f is capable of degrading single and double stranded RNA to mono, di, or trinucleotides, synthetic lin-4 levels were almost

undetectable after the RNase I_f treatment (Figure 6). The RNase treatment of lin-4 resulted in a significant fall of 14 Cqs (a 16,384 fold drop in miRNA number) (Figure 6). This is in contrast to the ~2-4 fold reduction in soybean and rice miRNA levels when cooked materials were transferred and incubated in the simulated digestion system for 75 min (Figure 3). These results also confirm the ability of the TaqMan miRNA assays to work with samples with varied levels of degradation.

4. Concluding remarks

By choosing a more simplified methodology for dietary plant miRNA evaluation, our results reveal for the first time the robustness of plant-derived miRNAs during food processing and cooking. In addition, this study found a continual survivability of plant miRNAs in a simulated digestion system for over an hour without any significant decrease in their levels. In contrast, similar treatments on synthetic cel-lin-4 miRNA showed instability of the synthesized miRNA in the adverse chemical and physical conditions during processing, cooking, and simulated digestion. We believe that this is the first demonstration of plant miRNA stability during processing, cooking and digestion of plant foods. This paper tries to elucidate for the first time the bioavailability of plant miRNA content that occurs prior to any absorption or transport into the blood stream. We consider our data an important contribution for future studies of these molecules *in vivo* for defining the potential of plant-derived miRNAs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest are disclosed.

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Figure legends

Figure 1: The miRNA levels in soybean during a series of experimental stages. **1A**. Total RNA Experion profile of the raw soybean seeds showing good quality of the RNA extract with an RQI of 8. **1B**. The levels of soybean miRNAs in raw, soaked, and cooked beans along with those levels of miRNAs released in the RNase-free water used for soaking and cooking show significant difference ($p<0.05$) between each group, based on ANOVA and post-hoc testing. The lower the Cq value, the higher the

miRNA levels. *denotes that the miRNA levels in the soaked bean, cooked bean, and cooked water are significantly higher ($p<0.05$) than the raw bean control.

Figure 2. The levels of miRNAs in raw rice and cooked rice along with those levels of miRNAs released into the RNase-free water used for cooking show significant difference ($p<0.05$), between each group. The lower the Cq value, the higher the miRNA levels. *denotes that Osa-miR166 and 167 levels in rice are significantly high ($p<0.05$) in raw rice control compared to the treated groups based on ANOVA and post-hoc testing.

Figure 3. The miRNA levels in food materials during early digestion. **3A**. The miRNA levels of soybean in the simulated digestion system from cooked material (0 min) until 75 min of digestion. miR167 and miR166 show consistent levels from 15 min until 75 min of early digestion and comparable Cq values throughout early digestion for 75 min to the respective values at 0 min time point. The miR168 levels show least difference at each time point in the simulated digestion system compared to 0 min. **3B**. miRNA levels in rice in cooked rice throughout the early digestion for 75 min in the simulated gut. miR167, miR166 and miR168 show consistent levels from 15 min until 75 min of early digestion and comparable Cq values throughout early digestion for 75 min to the respective values at 0 min time point. Sampling and analysis were carried out three independent times (n = 3), and error bars on each column, reflect SEM. Sampling and analysis were carried out three independent times (n = 3), and error bars on each column, reflect SEM. The lower the Cq value, the higher the miRNA levels.

Figure 4. RNA integrity and levels in processed soybean samples. **4A**. Total RNA profile of the soaked soybean. **4B**. Total RNA profile of the cooked soybean. **4C**. LEA mRNA transcript levels in soybean which had undergone a series of treatments along with control raw bean sample. The lower the Cq the higher the LEA mRNA level. *denotes that the control raw bean has the highest LEA mRNA level ($p<0.05$) compared to the bean samples which had undergone various treatments, based on one-way ANOVA and post-hoc testing. Sampling and analysis were done three independent times (n = 3), and error bars on each column, reflect SEM.

Figure 5. Comparison of synthetic miRNA cel-lin-4 and plant miRNA for their stability during soaking, cooking, and digestion at different time points. The synthetic molecules show significantly ($p<0.05$) high Cq values in the treated samples compared to soybean miRNAs based on one-way ANOVA and post-hoc testing. The lower the Cq the higher the miRNA levels. *denotes that the cel-lin-4 levels in the treated samples are significantly ($p<0.05$) lower than the untreated cel-lin-4 level. Sampling and analysis were carried out three independent times (n = 3), and error bars on each column, reflect SEM.

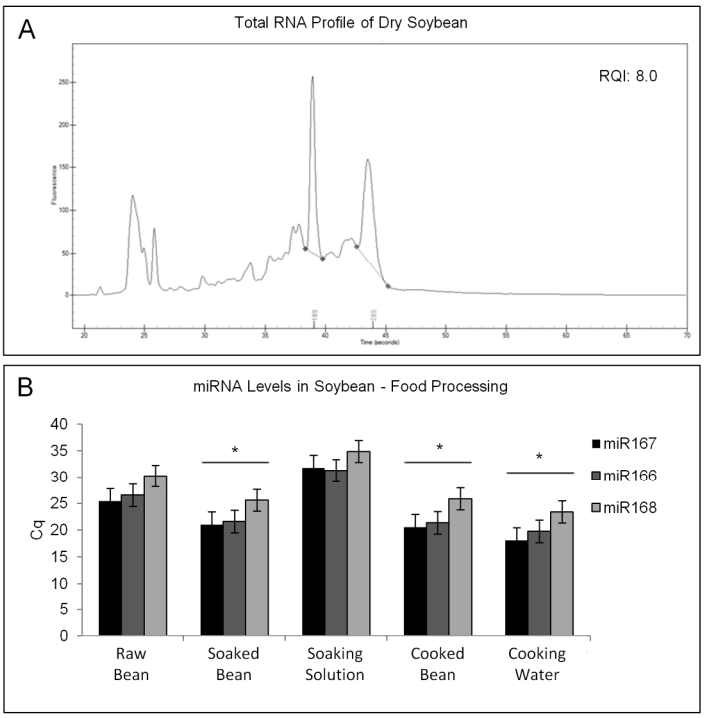
Figure 6. Comparison of heat and RNase treatments on synthetic miRNA. RNase treatment had significantly ($p<0.05$) higher rate of degradation than heat treatment on synthetic miRNA molecules. The lower the Cq the higher the miRNA levels are. * denotes that Boiling and RNase treatments on the synthetic cel-lin4 miRNA shows the significant difference ($p<0.05$) in the degree of degradation while the control Lin-4 remain intact. Sampling and analysis were carried out three independent times (n = 3), and error bars on each column, reflect SEM.

Table 1: Copy number of miRNAs per milligram of soybean (extrapolated from dry weight), calculated using a cel-lin-4 standard curve.

	miRNA Copy Number in Soybean (Mean ±SEM)					
	Raw Bean	**Soaked Bean**	**Cooked Bean**	**15min Digest**	**45 min Digest**	**75min Digest**
miR167	$4.4x10^{7} \pm 1.2x10^{7}$	$9.3x10^{8} \pm 1.6x10^{8}$	$1.1x10^{9} \pm 3.3x10^{8}$	$2.7x10^{8} \pm 5.0x10^{7}$	$2.9x10^{8} \pm 5.3x10^{7}$	$2.9x10^{8} \pm 7.9x10^{7}$
miR166	$2.7x10^{7} \pm 4.4x10^{6}$	$5.7x10^{8} \pm 1.1x10^{8}$	$6.9x10^{8} \pm 1.5x10^{8}$	$1.8x10^{8} \pm 3.6x10^{7}$	$2.0x10^{8} \pm 4.3x10^{7}$	$2.0x10^{8} \pm 4.5x10^{7}$
miR168	$1.6x10^{6} \pm 4.8x10^{5}$	$3.7x10^{7} \pm 9.6x10^{7}$	$3.2x10^{7} \pm 5.6x10^{6}$	$1.9x10^{7} \pm 4.9x10^{6}$	$1.6x10^{7} \pm 4.6x10^{6}$	$1.4x10^{7} \pm 3.0x10^{6}$
Lin-4	$3.9x10^{4} \pm 6.1x10^{3}$	$3.4x10^{4} \pm 8.6x10^{3}$	$7.9x10^{4} \pm 1.7x10^{4}$	$9.3x10^{4} \pm 1.7x10^{3}$	$1.9x10^{5} \pm 3.9x10^{4}$	$1.0x10^{5} \pm 3.3x10^{3}$

Sampling and analysis were carried out three independent times (n = 3).

Figure 1



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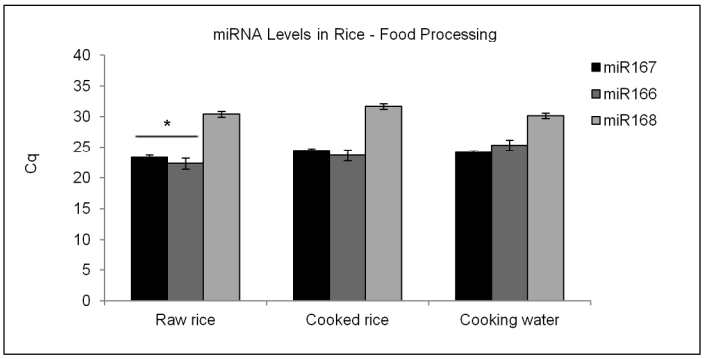

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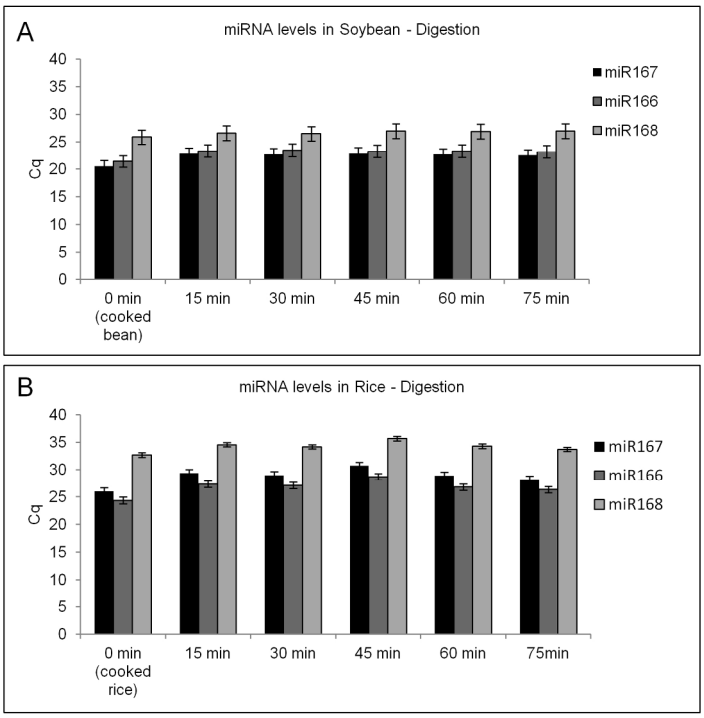

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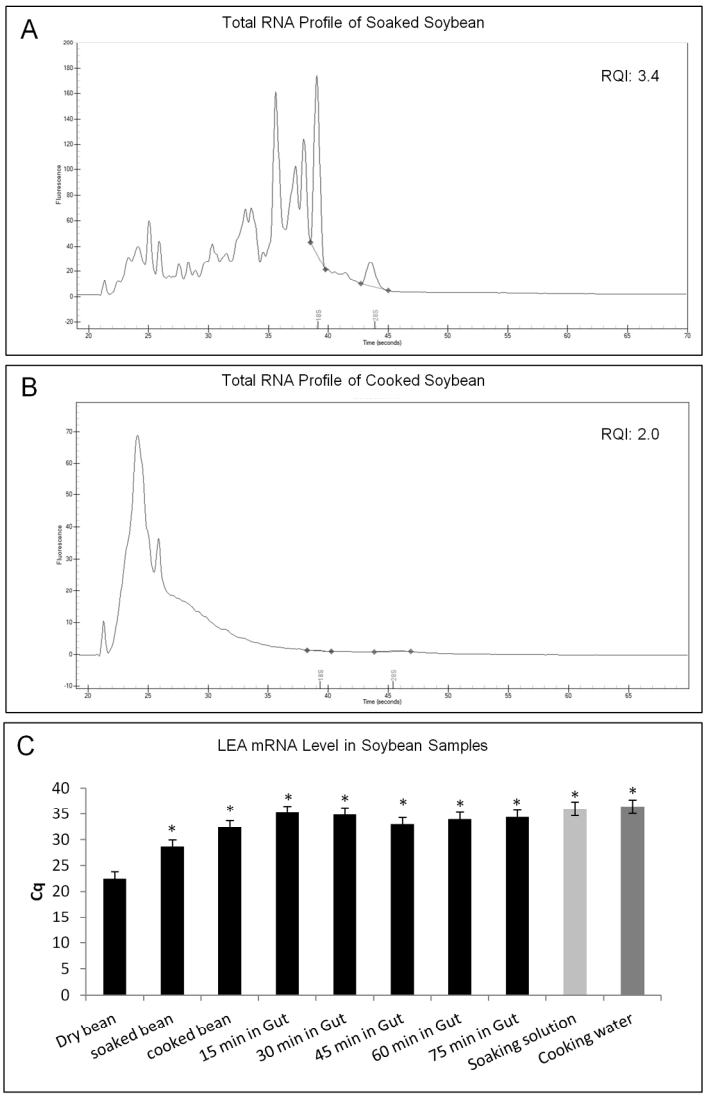

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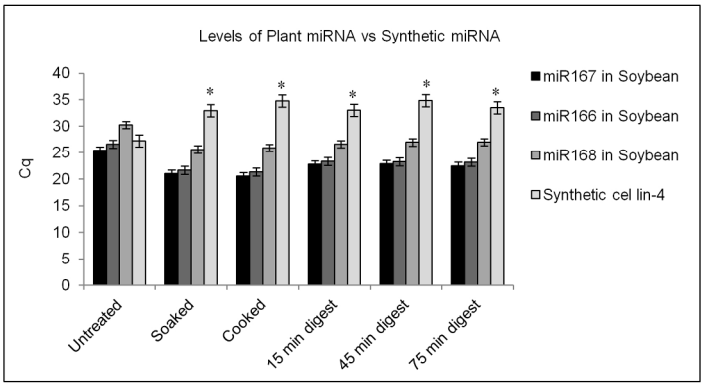

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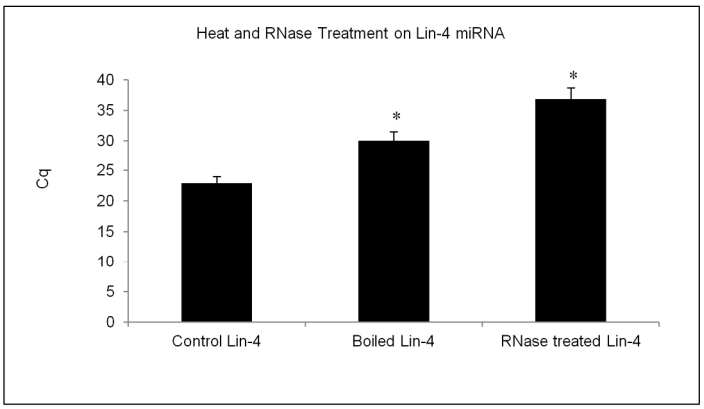

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