Indian Journal of Experimental Biology
Vol. 58, July 2020, pp. 474-486

A combination process including ionizing radiation for hygienization and shelf life extension of leafy vegetables

HD Khade[1,3], Sachin N Hajare[1], KSS Sarma[2] & Satyendra Gautam[1,3]*

[1]Food Technology Division, Bhabha Atomic Research Centre, Mumbai–400 085, Maharashtra, India
[2]Board of Radiation and Isotope Technology, BRIT / BARC Vashi Complex, Sector 20, Vashi, Navi Mumbai-400 703, Maharashtra, India
[3]Homi Bhabha National Institute, Anushakti Nagar, Mumbai-400 094, Maharashtra, India

Received 08 February 2019; *revised 05 June 2019*

Raw leafy greens are commonly associated with global foodborne outbreaks due to pathogenic contaminants. In the current study, greens, such as spinach (S*pinacia oleracea* L.), coriander *(Coriandrum sativum* L.) and mint (*Mentha spicata* L.) showed presence of coliforms (including *E. coli*) along with other aerobic microbes, yeast and molds. These vegetables mostly consumed in raw or culinary purpose, and hence increase the chances of food borne illnesses. Moreover, the leafy greens are perishable. In this context, we optimized a combination process including radiation treatment to achieve hygienization and shelf life extension of these leafy green vegetables. The combination treatment comprising potable water wash, chlorination (NaOCl-200 ppm) followed by irradiation (2 kGy using electron beam or gamma) was developed, and the processed samples showed keeping quality up to 15 days at 4-6°C, whereas control samples spoiled within two days. The treatment resulted in coliform count below detection level while retaining the nutritional, phenolic content and organoleptic qualities. Thus, the combination treatment could ensure safety, keeping quality enhancement of perishable leafy greens and to control global food outbreaks. Electron beam over gamma processing found to be a commercial viable option due to its high throughput and equal efficacy in microbial decontamination.

Keywords: Chlorination, Coliforms, Coriander, *Coriandrum sativum*, Greens, *Mentha spicata*, Microbial decontamination, Mint, Shelf life, Spinach, S*pinacia oleracea*

Leafy greens have become an integral part of the diet in today's world due to their prophylactic and nutritional values and low-calorie content. However, due to their association with soil and post harvest handling, the product often gets contaminated with high microbial load and even pathogens leading to foodborne outbreaks if consumed raw[1]. In such leafy agri-produce, microorganisms including coliform are introduced during their cultivation through irrigation, fertilizer application, post-harvest processing and distribution[2].

Over the last few years, there has been an alarming concern about the microbiological safety of the leafy greens. As an indication of this, new research and surveillance programs have been initiated worldwide. World Health Organization (WHO) too has declared food safety as an issue of prime importance[3]. Leafy agri-produce has been reported as one of the major sources of foodborne contaminations. According to the CDC (Centre for Disease Control and Prevention), USA, around 46% of reported foods borne outbreaks are linked to the fresh fruits and leafy vegetables. USA public health department reported 45 food borne disease outbreaks, resulting in 2429 cases of illness, 50 hospitalizations, and 2 fatalities between the period 1996–2016[4]. CDC reported that about 112000 food borne illnesses occur annually in the United States due to eating contaminated leafy greens with 12.8% hospitalization rate[5]. Outbreaks associated with leafy greens have been documented in the other parts of world too due to the large volume of international trade. Among the 790 outbreaks, Noro virus was reported to be the most common contaminant which accounted for around 42% outbreaks, followed by *Salmonella* causing ~30% outbreaks[6]. Other major bacterial pathogens associated with leafy greens causing food borne diseases include *Shigella* spp., *E. coli* O157:H7, *Yersinia* spp., *Campylobacter* spp., *Listeria* and *S. aureus* spp[7,8]. India is the second largest producer of fresh vegetables in the world after China and contributes for about 15% of the world's vegetables production[9]. Over 95% of India's fresh agri-

*Correspondence:
Phone: +91 22 25595379; Fax: +91 22 25505151
E-mail: sgautam@barc.gov.in (SG); khadehd@barc.gov.in (HDK)

produce is handled by the unorganized sector and it involves many intermediates before reaching to the customers[9]. This makes such produce more vulnerable to microbial contaminations. A study conducted also revealed the presence of *E. coli*, *Salmonella* spp., *Listeria* spp., on salad vegetables[10]. Fresh produce like chicory, celery and lettuce are also very prone to post harvest losses due to *Pseudomonas* spp. rot[11]. Presence of *Listeria* spp. in the vegetables such as lettuce, tomato and cabbage have been reported in various countries[12]. Ionizing radiations such as gamma radiation and electron beam have been reported to have potential to effectively eliminate pathogens, reduce the bio-burden and extend the shelf life of fresh produce[13]. This process is non-thermal and it does not increase the temperature of the products, resulting in retention of quality. Both the radiation sources ^{60}Co and electron beam are internationally approved for food processing[14].

More than 60 countries are using radiation processing technology for preservation of foods. In India, radiation processing of food is approved by FSSAI (Food safety and Standards Authority of India) as per revised latest notification FSSAI, Ministry of Health and Family Welfare Gazette Notification Part III-Section 4, (2016). Internationally IAEA (International Atomic Energy Agency) has approved this radiation treatment for processing of foods. Other international agencies like WHO/FAO also endorsed this technology for food safety and security. Other applications of low dose electron beam have also been studied in the field of agriculture[15]. In India, commercially food processing facilities are operational in private as well as government sectors for radiation processing of agriculture commodities like mango, onion, potato and other agri-produces[16]. Till date however, there are few reports on use of combination treatment using electron beam for sanitization of leafy vegetables. In the present study, we tried to develop a combination process including gamma or electron beam radiation to achieve hygienization and shelf life extension of fresh leafy agri-produce. Also, it aims at inactivation of *E. coli* present in leafy produce using optimized combination process. Along with gamma radiation, alternate viable option of e-beam irradiation is also chosen for radiation processing of fresh agri-produce.

Materials and Methods

Procurement, processing of samples and treatment condition

Freshly harvested varieties of spinach (cv. Semi-Savoy), coriander (cv. Co-2) and mint (cv. Japanese mint/menthol mint) were selected for the study. Farm fresh mature and dark leafy greens were collected from region of Nashik district of Maharashtra state, India. Roots of the plants were cut and the shoots were thoroughly cleaned and treated first with potable water for 5 min (designated as T1) and then treated with sodium hypochlorite (200 ppm) for the same time (5 min) (designated as T2). Later these samples were air dried for 30 min and were packed in circular tray (5 cm total thickness for EB processing (2 kGy) with cellulose based material to retain the moisture and covered with PVC film to maintain the freshness of the produce (Suppl. Fig. S1. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*). For pathogen screening (*E. coli* and *Salmonella typhimurium*), samples (around 200 g each in replicates) were collected and kept at low temperature (6–8°C) till analysis which was performed within 24 h of collection (Suppl. Table S1).

Irradiation and combination treatment

Gamma irradiation

Ranges of doses from 1-3 kGy were used for dose optimization for reduction in microbial load. Gamma radiation processing of the packaged samples were carried out at the dose of 2 kGy (D_{min}) as per the US code of federal regulation (21 CFR 179.26) as well as the new food rules notified by Government of India (FSSAI)[17] for fresh vegetables in a ^{60}Co based food package irradiator (Gamma radiation dose rate 30 Gy/min) at the institute. This treatment was designated as T3. Dosimetry was performed by ceric-cerous dosimetry system[18]. The DUR (Dose Uniformity Ratio) (D_{max}/D_{min}) were found to be 1.4.

Electron beam irradiation

Electron beam radiation processing of packaged samples was carried out at the institute's e-beam facility at the doses of D_{min}= 2 kGy. This treatment was designated as T4. Pre-calibrated BF3 film dosimetry system was used to measure the absorbed dose[19].

Combination process (CP)

Combination process was evaluated included washing steps and irradiation. In the first combination, the vegetable samples were washed either with potable water, filtered water or distilled water (T1) for 5 min and then were washed with sodium hypochlorite (200 ppm, pH 6.6-7) for 5 min (T2). Later the samples were air dried for 30 min, packed in trays with cellulose based material as moisture supplier and irradiated at

2 kGy using gamma irradiation (T3), this combination treatment (T1+T2+T3) was designated as Combination process 1 (CP1). Similarly, in second combination treatment, the same above process T1 and T2 was followed except that the samples were irradiated at 2 kGy using electron beam (T4) instead of gamma. This combination treatment (T1+T2+T4) was designated as CP2.

Microbial analysis

Microbiological analysis of vegetable samples was carried out for (APC) aerobic plate count, (PC) presumptive coliforms and (YMC) yeast and mould count. The samples were analysed using U.S. FDA Bacteriological Analysis Manual with some modification[20]. Briefly, in 225 mL of saline (0.85%), 25 g of the sample was suspended and was serially diluted (1:10) in the same. Total aerobic bacterial count was analyzed by standard plate count (SPC) on plate count agar (Hi Media Laboratories Pvt. Ltd., Mumbai, India) by pour plate technique followed by incubation at 37°C for 48 h. Similarly, estimation of presumptive coliforms was carried out using Violet Red Bile Agar (VRBA, pH 6.8) and YMC was carried out using potato dextrose agar (PDA) (pH 4.5) (Hi Media Laboratories Pvt. Ltd., Mumbai, India). The VRBA (violet red bile agar) plates were incubated at 37°C for 24 h, whereas PDA (potato dextrose agar) plates at 26°C for 5 days and counts were expressed as CFU/g.

Inoculated packed studies

Inoculated packed studies were carried out according to the procedure described by Behrsing *et al.*[21] with slight modification. Most commonly occurring pathogens *Salmonella* and coliforms (indicator organism for faecal contamination) were used in the present study. Both the organisms were grown overnight and a suspension of 10^7 cells/mL were prepared. Gamma radiation (4 kGy) hygienized vegetable samples (10 g) were dipped in aforesaid suspensions separately for 5 min and air dried in laminar flow cabinet. After drying, numbers of cells adhering to the vegetable samples were analyzed. These samples were subsequently treated with the combination treatments (CP1 as well as CP2) and the cell count was taken.

Efficacy of individual treatments on pathogenic load

The efficacy of individual treatments (T1-T4) was assayed on presumptive pathogens (3 isolates of each presumptive coliforms and *Salmonella*). Single colony of the presumptive pathogen was inoculated in 20 mL of MacConkey's and Lactose Broth (LB) and grown at 37°C for overnight for enrichment. Next day, 1.0 mL culture was centrifuged and washed with 0.85% NaCl. Subsequently, about 4 log CFU/mL cells were treated with single treatment (T1-T4) in aqueous system. After the treatment, the cells were serially diluted from 10^{-1} to 10^{-4}. All the dilutions were plated on LA (Luria agar, Hi Media Laboratories Pvt. Ltd., Mumbai, India) plates, incubated at 37°C for overnight. Next day, the count was taken and expressed as log CFU/ mL.

Screening and confirmation of coliforms

Microbiological analysis was carried out within 72 h of receipt of samples[20]. For leafy greens, representative leaves from the different part of the bunch were used to get up 25 g samples, which were mixed in a stomacher for 2 min in 225 mL of Brain Heart Infusion (BHI) enrichment broths, the suspension was further incubated up to 3 h at 37±0.5°C. After enrichment the contents were added in 250 mL of Tryptone Phosphate (2X) broth and incubated at 37±0.5°C for 24 h. A loopful of sample from enriched broth was withdrawn and streaked on Eosin Methylene Blue (EMB) agar plate / VRBA agar plate and were incubated at 37°C for 24 h. Colonies with dark centered and flat, with or without metallic sheen were selected. Five putative colonies were transferred from each EMB plate to plate count Agar slants and incubated for 18-24 h at 37°C and preserved for further use. Colonies streaked on EMB and MacConkey agars were incubated for 18-24 h at 37°C and observed for colony morphology. Putative isolates were categorized as presumptive coliforms on basis of colony morphology. Further confirmation was carried out by IMViC test.

Screening and confirmation of *Salmonella* spp.

The detection of *Salmonella* was carried out according to the standard method mentioned in the US FDA's Bacteriological Analytical Manual[20]. Leafy vegetable sample (25 g) were blended with Lactose broth 225 mL (Hi Media Laboratories Pvt. Ltd., Mumbai) and incubated for 24 h at 37°C. The pre-enriched sample was then transferred into tubes containing 9 mL of tetrathionate (Hi Media Laboratories Pvt. Ltd, Mumbai, India) and Rappaport Vassiliadis (Hi Media Laboratories Pvt. Ltd. Mumbai) broths and incubated at 42.5°C in water bath. Tubes that showed distinct turbidity were then streaked on xylose lysine deoxycholate (Hi Media Laboratories Pvt. Ltd., Mumbai, India) and bismuth sulphite

(Hi Media Laboratories Pvt. Ltd., Mumbai, India) plates. Isolates were categorised as presumptive *Salmonella* based upon colony morphology and further confirmed by IMViC test.

Confirmation of presumptive pathogens by PCR amplification of specific genes

Confirmation of presumptive pathogens was carried out by colony PCR, amplification of specific genes for *E. coli* and *Salmonella* Typhimurium. PCR conditions were set as: Initial denaturation: 5 min, 94°C, and 35 numbers of cycles of annealing: 55.2°C for 10 s, extension: 72°C for 1.0 minute and denaturation at 94°C for 45 s[22]. For presumptive coliforms, Universal Stress protein –A (*Usp A*) gene of *E. coli* was selected while for presumptive *Salmonella* isolates, Invasion-A (*Inv A*) gene of pathogenic *Salmonella* was selected. The forward and reverse primers for *Usp A* gene were usp A F 5′ CCGATACGCTGCCAATCAGT 3′ and usp AR 5′ ACG CAG ACC GTA GGC CAG AT 3′, respectively while for *Inv A* gene, the primers were S139F 5′ GTG AAA TTA TCG CCA CGT TCG GGC AA 3′ and S141R 5′ TCA TCG CAC CGT CAA AGG AAC C 3′, respectively[23].

Organoleptic analysis

Organoleptic evaluation was performed by experts using 9-point hedonic scales (9-like extremely, 8-like strongly, 7-like very well, 6-like fairly well, 5-like moderately, 4-like slightly, 3-dislike slightly, 2-dislike moderately, 1-dislike extremely) in an independent partitioned compartment in a sensory laboratory. Parameters evaluated were appearance, colour, flavour, texture, taste, after taste and overall acceptability. Samples cooked in boiling water for 10 min were used for flavour, taste and after taste parameters where as raw samples were used for appearance, colour and texture analysis[24].

Colour analysis

Colour of the vegetable samples was measured through direct reading on the central region by reflectance using Minolta CM-3600d Spectrophotometer (Konica Minolta, Japan). Six measurements were taken for each sample. The visible spectrum (360-780 nm) were recorded ($\Delta\lambda$ = 10 nm). Illuminant D_{65} and 10° Observer was considered as references. The L*, a* and b* CIELAB parameters were obtained[25].

Preparation of vegetable extract and biochemical analysis

The leaves were washed under tap water and crushed using mortar and pestle with liquid nitrogen into fine powder which was used for biochemical analysis. The biochemical analyses were carried out using either powder or 1% methanolic extract. Chlorophyll content and total phenolics were analyzed by Arnon's and Folin-Ciocalteu methods, respectively as detailed below.

Determination of total soluble phenolics

Total soluble phenolics in the vegetable extracts were determined according to the Folin-Ciocalteu procedure[26]. Appropriately diluted methanolic extract was mixed with Folin-Ciocalteu reagent (0.2 N) and allowed kept at ambient temperature (32±2°C) for 5 min. Sodium bicarbonate solution (6%, 0.75 mL) was added to the mixture and incubated at ambient temperature (28°C) for 90 min. Absorbance of the solution was measured using a spectrophotometer at wavelength 725 nm. A calibration curve was prepared using standard Gallic acid and the results were expressed as GAE (mg Gallic Acid equivalent/g)[26].

Chlorophyll content determination

Total chlorophyll content was estimated by the method described by Arnon[27]. This method is based on the principle that the chlorophylls are extracted in organic solvents and then the absorption of extracted chlorophylls is measured at 663 and 645 nm (absorption maxima for chlorophyll 'a' and 'b', respectively). Fresh leaf material (20 mg) was homogenized in microfuge tube and mixed with 1.0 mL DMSO. The suspension was centrifuged and again DMSO (1 mL) was added to the pellet and re-extracted. Absorbance of blank and samples was measured at 645 and 663 nm and content of total chlorophyll was calculated according to the following equation:

Total Chlorophyll (g /L) = $0.0202\ A_{663nm} + 0.00802\ A_{645nm}$[27].

Physical, biochemical and nutritional analysis

Nutritional analysis of leafy vegetable samples (control as well as combination treated) was carried out. The parameters quantified were moisture; ash; total protein; fat content; carbohydrates; total sugars; dietary fibre; vitamins A, B1, B2 and C. The analyses were carried out according to either methods of BIS (Indian Standard methods) or AOAC (Association of Official Analytical Chemists). Briefly, moisture content of the vegetable samples was determined according to the method of Bureau of Indian Standards (BIS) by drying in the oven. Total ash content was determined according to the standard AOAC protocol[28]. For ash content, samples were incinerated in a muffle furnace at 550±20ºC for about 6 h until grey

ash resulted and subsequently the ash content was determined. Total fat content was estimated by the method of BIS using soxhlet extraction apparatus[29]. Total reducing sugar content of vegetable samples was determined according to the AOAC protocol[30] based on redox titration using freshly prepared Fehling's solution while total protein content was determined using a BIS protocol[31] based on Kjeldahl method. Carbohydrate content of vegetable samples was determined by calculating the difference in percentage after deducting the percentage values of fat, protein, ash and moisture from the total (100%). Energy content of vegetable samples was determined by calculation, after estimating the protein, sugar, fat and carbohydrate contents of the sample. Niacin was estimated by using a BIS protocol[32]. In this method, niacin/nicotinic acid react with cyanogen bromide to form a pyridinium compound which undergoes reorganization and yields derivatives that couples with aromatic amines, gives coloured compounds which is quantitatively estimated photometrically. Total ascorbic acid content of vegetable samples was determined by titrimetric process using dye 2,6-dichlorophenol indophenol according to the BIS method[33] while vitamin A content was determined by a spectrophotometric method as per BIS method[34]. Precautions were taken to protect the vitamin A content in the flask from direct light. Riboflavin estimation was carried out using a BIS method[35]. In this the vitamin was extracted in hydrochloric acid and then estimated by measuring the fluorescence at 440 nm.

Statistical analysis

The data were analyzed using the software version v6.1052 (B232) of Origin 6.1 (OriginLab Corp., Northampton, Mass., U.S.A.) at 0.05 level of significance. Mean and standard deviations for all the results, were calculated, whereas (ANOVA) one-way analysis of variance was used to find out significant differences within the samples during storage as well as among treatments.

Results and Discussion

Combination process resulted in improved hygiene of leafy vegetables

New rule notified by the Food Standards and Safety Authority, Government of India and 21CFR-179.26 federal regulation of USA also considered for optimization of the radiation dose. Fresh spinach, coriander and mint samples were trimmed to remove the root portion, and then washed with potable water, air dried, packed in different packaging conditions, and irradiated at doses of 0, 1, 2, 2.5 and 3 kGy. In case of 1 kGy dose, there was no significant decrease in coliform load, whereas 2 kGy dose reduced the coliform count to below detectable level. Hence, 2 kGy was selected for detailed studies. Further higher doses of 2.5 and 3 kGy showed significant negative effect on sensory quality and could not retain the sensory scores.

Besides radiation treatment minimal processing including washing with potable water followed by washing with routinely used sanitizer (NaOCl) was also included in the process as these treatments are required to ensure the good level of hygiene upon treatment as well as during storage period. Also found that in samples treated with less than 2 kGy of radiation dose coliforms were still present and in more than 2 kGy treated samples although coliforms were undetectable but the organoleptic attributes were not well retained. Leafy greens were treated with potable water (5 min), then dipped in sodium hypochlorite (200 ppm, 5 min) and irradiated at 2 kGy. Amongst the sanitizers NaOCl (at the concentration of 200 ppm) was preferred because it is one of the most commonly used sanitizer's worldwide[36]. For retaining the freshness during storage at low temperature (4-6°C), moist packaging in trays was optimized where the trays were covered with PVC film to reduce the moisture loss. Such produce was found to be visibly acceptable for the minimum storage period of 15 days when stored at 4-6°C and RH- 85-90%. On the other hand, samples stored at ambient temperature (30-32°C) showed shelf life of one to two days only. The findings of the inoculated pack study conducted with *E. coli* and presumptive *Salmonella* spp. indicated the cell count of both these organisms reached to below detectable level upon CP1 as well as CP2 treatments (Suppl. Table S2).

Potable water washing resulted in required hygiene level of the product

In the above said combination process further question was asked whether replacing potable water with filtered or distilled water can be useful. In fresh spinach control sample, TAPC was observed to be around 8 log CFU/g, while YMC PC were around 6 log CFU/g (Table 1). Potable water wash during combination treatment was found to be sufficient to reduce the count below permissible limit and hence to make the process economic viable potable water was preferred over filtered or distilled water (Table 2).

Table 1— Microbial profile of leafy vegetables (storage at 4-6°C) subjected to combination process (Log CFU/g)

Treatments	Total aerobic plate count (TAPC) Storage period (days)					Yeast mould count (YMC) Storage period (days)					Presumptive coliform (PC)
	1st	3rd	7th	13th	15th	1st	3rd	7th	13th	15th	Day 1st to 15th
Spinach control	$7.5\pm1.4^{x,m}$	NA	NA	NA	NA	$5.6\pm2.1^{x,n}$	NA	NA	NA	NA	5.7 ± 2.2^{x0}
T3	$4.6\pm1.2^{y,m}$	$4.7\pm1.6^{x,m}$	$4.9\pm2.1^{x,m}$	$4.9\pm2.5^{x,m}$	$5.5\pm2.1^{x,n}$	$1.9\pm0.8^{y,o}$	$2.5\pm1.1^{x,p}$	$2.6\pm1.1^{x,p}$	$2.9\pm1.7^{x,p}$	$3.6\pm1.2^{x,q}$	<detn. levl.
CP1	$1.2\pm0.8^{z,m}$	$1.8\pm0.5^{y,m}$	$2.3\pm0.8^{y,n}$	$2.4\pm1.3^{y,o}$	$2.9\pm1.3^{y,o}$	$1.4\pm0.6^{z,p}$	$1.6\pm0.9^{y,q}$	$1.7\pm0.7^{y,q}$	$1.9\pm0.6^{y,q}$	$2.9\pm1.3^{y,r}$	<detn. levl.
T4	$4.9\pm2.1^{a,m}$	$4.9\pm1.6^{z,m}$	$4.9\pm1.1^{z,m}$	$5.6\pm2.1^{z,n}$	$5.9\pm2.6^{z,n}$	$2.4\pm1.2^{a,o}$	$2.6\pm1.5^{z,p}$	$2.8\pm1.5^{z,p}$	$2.9\pm1.2^{z,p}$	$3.4\pm1.6^{z,q}$	<detn. levl.
CP2	$1.8\pm0.3^{b,m}$	$1.8\pm0.9^{a,m}$	$1.9\pm0.8^{a,m}$	$2.6\pm0.8^{a,n}$	$2.9\pm1.7^{a,n}$	$1.9\pm0.7^{b,o}$	$1.9\pm0.7^{a,o}$	$1.5\pm0.8^{a,o}$	$2.2\pm1.4^{a,p}$	$2.8\pm1.1^{a,q}$	<detn. levl.
Coriander control	$7.6\pm1.5^{c,m}$	NA	NA	NA	NA	$5.5\pm2.1^{c,n}$	NA	NA	NA	NA	4.7 ± 0.2^{yo}
T3	$4.6\pm1.4^{d,m}$	$4.6\pm1.3^{b,m}$	$4.8\pm1.7^{b,m}$	$4.9\pm1.1^{b,m}$	$5.4\pm2.4^{b,n}$	$3.4\pm1.3^{d,o}$	$3.6\pm1.4^{b,o}$	$3.8\pm1.2^{b,p}$	$4.2\pm1.7^{b,q}$	$4.8\pm2.2^{b,r}$	<detn. levl.
CP1	$1.9\pm0.6^{e,m}$	$2.7\pm1.4^{c,n}$	$2.9\pm0.7^{c,n}$	$2.9\pm2.2^{c,n}$	$2.9\pm1.3^{c,n}$	$1.1\pm0.6^{e,o}$	$1.8\pm0.8^{c,p}$	$1.8\pm0.9^{c,p}$	$2.0\pm0.8^{c,p}$	$2.2\pm1.3^{c,q}$	<detn. levl.
T4	$4.5\pm1.3^{f,m}$	$4.8\pm1.8^{d,n}$	$4.9\pm2.1^{d,o}$	$4.9\pm2.5^{d,p}$	$5.5\pm2.6^{d,q}$	$3.7\pm1.2^{f,r}$	$3.9\pm1.1^{d,r}$	$3.9\pm1.3^{d,r}$	$4.2\pm1.1^{d,s}$	$4.7\pm2.1^{d,t}$	<detn. levl.
CP2	$1.9\pm0.7^{g,m}$	$2.8\pm0.1^{e,n}$	$2.9\pm0.9^{e,o}$	$2.9\pm0.9^{e,p}$	$2.9\pm1.4^{e,q}$	$1.9\pm0.7^{g,r}$	$2.0\pm1.2^{e,r}$	$2.1\pm1.3^{e,r}$	$2.8\pm1.3^{e,s}$	$2.9\pm0.9^{e,t}$	<detn. levl.
Mint control	$7.1\pm1.9^{h,m}$	NA	NA	NA	NA	$5.3\pm2.2^{h,n}$	NA	NA	NA	NA	5.6 ± 0.1^{z0}
T3	$4.3\pm1.5^{i,m}$	$4.6\pm1.4^{f,n}$	$4.8\pm2.5^{f,o}$	$5.2\pm2.3^{f,p}$	$5.6\pm2.8^{f,q}$	$2.5\pm1.7^{i,r}$	$2.8\pm1.5^{f,r}$	$2.9\pm1.1^{f,r}$	$3.3\pm1.2^{f,s}$	$3.7\pm1.2^{f,t}$	<detn. levl.
CP1	$1.0\pm0.^{6j,m}$	$1.8\pm0.7^{g,n}$	$1.9\pm1.1^{g,o}$	$2.4\pm1.2^{g,p}$	$2.9\pm1.7^{g,q}$	$1.2\pm0.8^{j,r}$	$1.8\pm1.1^{g,s}$	$1.8\pm0.7^{g,s}$	$1.9\pm0.8^{g,s}$	$2.4\pm1.4^{g,t}$	<detn. levl.
T4	$4.6\pm1.4^{k,m}$	$4.8\pm1.6^{h,n}$	$4.9\pm2.2^{h,o}$	$5.4\pm2.2^{h,p}$	$5.6\pm2.1^{h,q}$	$2.5\pm1.5^{k,r}$	$2.7\pm1.6^{h,r}$	$2.9\pm1.2^{h,r}$	$3.2\pm1.1^{h,s}$	$3.7\pm1.5^{h,t}$	<detn. levl.
CP2	$1.9\pm0.8^{l,m}$	$1.9\pm0.8^{i,n}$	$1.9\pm0.9^{i,o}$	$2.8\pm1.1^{i,p}$	$2.9\pm1.4^{i,q}$	$1.8\pm1.1^{l,r}$	$1.8\pm0.8^{i,r}$	$1.8\pm1.6^{i,r}$	$1.9\pm0.9^{i,r}$	$2.4\pm1.1^{i,s}$	<detn. levl.

[Control- fresh samples non treated; T3, gamma irradiation (2 kGy); T4, electron beam irradiation (2 kGy); CP1, potable water wash + sodium hypochlorite treatment (200 ppm) + gamma irradiation (2 kGy); CP2, potable water wash + sodium hypochlorite treatment (200 ppm) + electron beam irradiation (2 kGy); NA, not available for analysis due to spoilage of sample (control); $^{x,y,z,a,b,c\ to\ j,\ k,\ l}$ Different letters across the column and $^{m,n,o\ to\ r,s,t}$ different letters across the rows indicates significant difference ($P \leq 0.05$) between the sample means as analyzed by one way ANOVA; CFU/g, colony forming unit per gram; and detn. levl., detection level]

Table 2 — Microbial profile of spinach (storage at 4-6°C) subjected to combination process

Treatments	Total aerobic plate count (TAPC) (Log CFU/g) Storage period in days					Yeast mould count (YMC) (Log CFU/g) Storage period in days					PC (Log CFU/g)
	1st	3rd	7th	13th	15th	1st	3rd	7th	13th	15th	Day 1st to 15th
Spinach control	$7.5\pm0.2^{a,h}$	NA	NA	NA	NA	$5.6\pm0.3^{a,i}$	NA	NA	NA	NA	5.7 ± 0.2^{aj}
(CP1) P	$2.2\pm0.2^{b,h}$	$2.6\pm0.1^{a,i}$	$2.9\pm0.3^{a,j}$	$3.4\pm0.2^{a,k}$	$3.7\pm0.3^{a,l}$	$2.4\pm0.2^{b,m}$	$2.6\pm0.3^{a,n}$	$2.7\pm0.2^{a,n}$	$2.9\pm0.1^{a,o}$	$3.9\pm0.3^{a,p}$	<detn. levl.
(CP2) P	$2.6\pm0.2^{c,h}$	$2.8\pm0.2^{b,h}$	$2.9\pm0.1^{a,h}$	$3.6\pm0.2^{a,i}$	$3.9\pm0.2^{a,j}$	$2.7\pm0.1^{c,k}$	$2.9\pm0.2^{b,l}$	$2.9\pm0.1^{b,l}$	$3\pm0.3^{b,m}$	$3.8\pm0.2^{b,n}$	<detn. levl.
(CP1) F	$1.9\pm0.3^{d,h}$	$2.0\pm0.2^{c,h}$	$2.3\pm0.2^{b,i}$	$2.5\pm0.1^{b,,j}$	$2.7\pm0.2^{b,k}$	$1.5\pm0.2^{d,l}$	$1.8\pm0.1^{c,m}$	$1.8\pm0.1^{c,m}$	$2.0\pm0.1^{c,n}$	$2.2\pm0.2^{c,o}$	<detn. levl.
(CP2) F	$1.9\pm0.1^{d,h}$	$2.1\pm0.3^{c,h}$	$2.4\pm0.1^{b,i}$	$2.6\pm0.3^{b,,j}$	$2.9\pm0.1^{c,,k}$	$1.9\pm0.1^{e,l}$	$2.0\pm0.2^{d,m}$	$2.1\pm0.3^{d,m}$	$2.4\pm0.2^{d,n}$	$2.5\pm0.1^{d,n}$	<detn. levl.
(CP1) D	$1.6\pm0.2^{e,h}$	$1.8\pm0.1^{d,i}$	$1.9\pm0.2^{c,i}$	$2.4\pm0.2^{c,,j}$	$2.9\pm0.3^{c,,k}$	$1.5\pm0.3^{f,l}$	$1.8\pm0.2^{e,m}$	$1.8\pm0.1^{e,m}$	$1.9\pm0.1^{e,m}$	$2.4\pm0.3^{e,n}$	<detn. levl.
(CP2) D	$1.9\pm0.1^{f,h}$	$1.9\pm0.2^{d,h}$	$1.9\pm0.1^{c,h}$	$2.8\pm0.1^{d,,i}$	$2.9\pm0.1^{c,i}$	$1.8\pm0.1^{g,j}$	$1.8\pm0.1^{f,j}$	$1.8\pm0.2^{f,j}$	$2.1\pm0.3^{f,k}$	$2.3\pm0.2^{f,l}$	<detn. levl.

[Control- Fresh samples non treated. (CP1) P, (potable water wash, sodium hypochlorite (200 ppm), 2 kGy Gamma); (CP2) P, (potable water wash, sodium hypochlorite treatment (200 ppm), 2kGy EB); (CP1) F, [filter (RO)] water wash sodium hypochlorite (200 ppm), 2 kGy Gamma); (CP2) F, (filter water wash, sodium hypochlorite treatment (200 ppm), 2kGy EB); (CP1) D, (distilled water wash, sodium hypochlorite (200 ppm), 2 kGy Gamma); (CP2) D, (distilled water wash, sodium hypochlorite treatment (200 ppm), 2kGy EB); NA, not available for analysis due to spoilage of sample (control); $^{a-g}$ Different letters across the column and $^{h-p}$ different letters across the rows indicates significant difference ($P \leq 0.05$) between the sample means as analyzed by one-way ANOVA; and <detn. levl., <detection level]

Samples treated by combination process and stored retained keeping quality up to 15 days at 4-6ºC

The optimized combination process (CP1) includes the treatments: T1, washing with potable water; T2, NaOCl (200 ppm, 5 min) wash for 5 min; and T3, Gamma radiation 2 kGy. Similarly, combination (CP2) was also explored were gamma irradiation 2 kGy (T3) were replaced with same radiation dose (2kGy) of electron beam (T4). Although the gamma irradiation is a very effective technology, the commercial viability of gamma radiation plant is always limited by the availability of food material to be irradiated. This is

because radiation source will keep on depleting whether it is used or not. On the other hand, compared to gamma, electron beam treatment is cost effective for two reasons. One the e-beam machine can be switched on or off as per the need and availability of food material and the second, its dose rate is very high (operational maximum capacity of 10 kGy per second) compared to gamma radiation. So, if produce density and penetration is not an issue, electron beam is considerably cost effective due to high throughput.

T1 and T2 treatment resulted in reduction of TAPC to around 6 log CFU/g, YMC to 4 log CFU/g and PC to 4 log CFU/g, respectively (Suppl. Table S3). Gamma radiation (2 kGy) (T3) reduced TAPC and YMC in spinach to around 5 log CFU/g and 2 log CFU/g, respectively, and PC to below detection level (Table 1). Presence of PC even after consecutive washes with potable water (T1) and sanitizer (T2) demanded the use of further effective hygienization treatment. Upon the electron beam treatment (2 kGy), TAPC and YMC reduced to around 5 log CFU/g and 2 log CFU/g, respectively and PC to below detection level in treated spinach samples (Table 1).

The CP1 process treatment of spinach resulted in reduction of TAPC to 1.2 log CFU/g, YMC to 1.4 log CFU/g and PC to below detection level in case of spinach. Similarly, CP2 treatment resulted in reduction of TAPC to 2 log CFU/mL, YMC to 2 log CFU/g and PC to below detection level (Table 1). Thus, both combinations CP1 and CP2 proved to be better hygienization processes. During the storage of CP1 and CP2 treated samples at low temperature 4-6°C, there was one log cycle increase in both TAPC and YMC till 15 days in all the treated samples. However, PC counts remained below detectable level in similar storage condition. Similar decrease of 4 log cycles was observed by Palekar *et al*.[37] in vegetable when inoculated with *Salmonella enteric* serotype Poona and irradiated with EB at 1.5 kGy. Similar trend in reduction of microbial load in coriander (*Coriandrum sativum* L.) and mint (*Mentha spicata* L.) was observed upon CP1 and CP2 treatments (Table 1). Similarly, Collazo *et al*.[38] observed 2 $\log_{10}$ cycle reduction in both *Listeria monocytogenes* and *Salmonella enterica* in 'Iceberg' lettuce after treatment with water assisted UV-C light (1 kJ/m^2). However, in case of baby spinach leaves, a combination of water assisted UV-C light (2 kJ/m^2) and peroxyacetic acid (40 mg/L) showed only 1 $\log_{10}$ cycle reduction in both *L. mono cytogenes* and *S. enterica* cell population, respectively[38]. In another study, Foley *et al*.[39] showed that when fresh cut iceberg lettuce packed in film bags and in combination with chlorination was exposed to radiation dose of 0.55 kGy, showed a 5.4 log reduction in *E. coli* count. This dose of irradiation did not cause any change in textural as well as other qualities of lettuce. Earlier study also showed that, 1 kGy dose significantly inactivated *Pseudomonas* spp. which were inoculated in baby spinach and romaine lettuce[40]. Such treated samples could be stored up to 9 days at 4°C without affecting the visual quality. In the present study, the minimum shelf life of commodities was 15 days, significantly more than earlier reports.

PCR amplification of *E coli* specific *usp A* gene indicated presence of *E coli* in produce

In initial screening presumptive coliforms were observed in all the sample lots. However, presumptive *Salmonella* were found in only two batches out of 52 batches (Suppl. Table S1). Among 45 isolates screened only two were found to be presumptive S*almonella* spp. However, PCR amplification of *Inv A* gene (specific to *Salmonella*) confirmed the strains to be *Salmonella* spp. negative.

PCR amplification of *uspA* gene of the presumptive coliforms was carried out. Out of these, 3 isolates from spinach samples procured from location / place code No. 5 and two samples of coriander procured from location/place code No. 6 showed amplification (884 bp) of *usp A* gene, confirming the isolates to be *E. coli* (Fig. 1). Further, serological confirmation of these *E. coli*

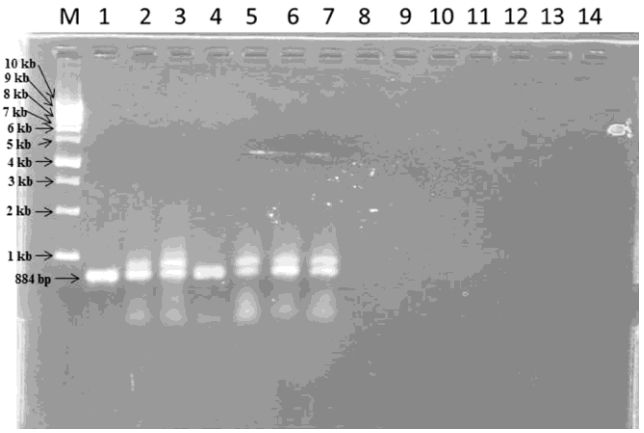

Fig. 1 — Amplification of specific gene *usp* A in presumptive *E. coli* isolates in leafy vegetable samples: PCR amplification (884 bp) of *usp A* gene in presumptive *E. coli* isolates in leafy vegetable samples. [M-,1 kb step up ladder Marker; Lane 1, Positive control; Lane 2, Positive control; Lanes 3-5, Spinach, from location No. 5; Lanes 6 & 7, Coriander from location No. 6; Lanes 8-10, Spinach from location No. 7; Lanes 11-13, Coriander from location No. 8; and Lane 14, Mint from location No. 9]

isolates was carried out by LK13– Hi *E. coli* O157™ Latex Test Kit (Hi Media Laboratories Pvt. Ltd. Mumbai, India). The kit confirms the isolates to be pathogenic by latex agglutination test. When tested, all the five isolates did not show agglutination indicating absence of O157 antigen/sub-spp. of *E. coli*. Thus, the *E. coli* isolates obtained from the spinach and coriander samples were O157 antigen negative. As these isolates showed amplification of *E. coli* specific *uspA* gene, it suggests that the faecal contamination could have occurred. Thus, the study revealed random presence of faecal coliforms on leafy vegetables sold in retail markets which is an issue of safety and concern. Therefore, processing of such leafy greens with CP1 or CP2 treatment was found to be essential.

Physicochemical properties

Samples treated by combination process retained freshness and visual characteristics

Moisture content in fresh leafy greens was found to be in the normal range which did not change significantly in CP1 & CP2 treated samples during the course of storage up to 15 days at 4-6°C. Similarly, the visual appeal of the combination processed leafy greens retained up to 15 days at low temperature storage.

Appearance of combination processed vegetables

As control samples of all the vegetables could not last more than 2 days, the colorimetric data for only day 1 was measured for all the control vegetable samples. The colour values in terms L* (lightness) for all the leafy green samples (control, CP1 and CP2 treated) varied in the range of 50-67 on day 1 (Table 3). The variation among the treatments in each commodity was due to the biological variation. In CP1 treated spinach samples, there was significant reduction in the lightness during storage on 13th and 15th day. CP2 samples did not show such reduction. In all other vegetables, there was no significant change in lightness of the CP1 and CP2 treated samples (Table 3). Greenness of all the leafy greens was in the range of −10 to −18. The significant change in the greenness values due to CP1 and CP2 was due to the biological variation among the samples. The greenness of the spinach samples remained unchanged till 7 days in both CP1 and CP2 samples while it reduced significantly on day 13th day and 15th in both the samples. In case of coriander and mint, the greenness remained unchanged throughout the storage period. The yellowness of all the leafy green samples was in the range of 18 to 24. In spinach, sample no significant difference ($P \geq 0.05$) occurred in terms of yellowness till 13th day of storage in both CP1 and CP2 treated samples.

CP1 treated samples showed significant reduction ($P \leq 0.05$) in yellowness on day 15 due to more browning. CP2 treated samples also showed the decrease in yellowness. However, the decrease was not statistically significant ($P \geq 0.05$). Similar trend of decrease in yellowness was observed in both CP1 and CP2 treated mint samples on day 13th and 15th of storage (Table 3). However, in one of the studies it was shown that there was no change in the visual appearance of spinach samples packed in perforated film bags and irradiated up to 4 kGy[41].

Thus, overall in all these vegetable samples, there was no pronounced effect of CP1 or CP2 treatment on the colour parameters were observed during storage of 15 days at 4-6°C. Similarly, in another study researchers did not find any significant change in colour parameters of irradiated (2 kGy) mint samples during the storage period of 12 days[42].

Table 3 — Colorimetric analysis of leafy vegetables treated with combination process and stored at 4-6°C

Treatment	Index/ Shade colour	Spinach				Coriander				Mint			
		Storage period (Days)											
		1st	7th	13th	15th	1st	7th	13th	15th	1st	7th	13th	15th
Control	L*	53±5^{m,a}	NA	NA	NA	52±2^{m,b}	NA	NA	NA	54±7^{m,c}	NA	NA	NA
CP1		61±6^{n,a}	59±6 ^{m,a}	48±5^{m,b}	48±4^{m,b}	56±5^{n,c}	53±5^{m,c}	51±3^{m,d}	51±5^{m,e}	56±6^{m,f}	61±6^{m,g}	50±5^{m,h}	50±5^{m,h}
CP2		56±6^{o,a}	53±5^{n,a}	55±6^{n,a}	50±6^{n,b}	57±5^{n,c}	54±4^{m,d}	52±5^{m,e}	50±5^{m,e}	58±2^{n,f}	55±6^{n,f}	58±6^{n,f}	53±7^{m,g}
Control	a*	-17±2^{p,a}	NA	NA	NA	-10±1^{o,b}	NA	NA	NA	-18±3^{o,c}	NA	NA	NA
CP1		-14±3^{p,a}	-14±2^{o,a}	-10±1^{o,b}	-10±2^{o,b}	-11±2^{p,c}	-10±1^{n,c}	-10±2^{n,c}	-10±2^{n,c}	-14±2^{p,d}	-10±2^{o,e}	-10±1^{o,e}	-10±4^{n,e}
CP2		-15±2^{q,a}	-13±2^{o,a}	-10±2^{o,b}	-10±1^{o,b}	-11±3^{q,c}	-11±2^{n,c}	-11±2^{n,c}	-11±1^{n,c}	-14±2^{q,d}	-14±2^{p,d}	-11±1^{o,e}	-11±2^{n,e}
Control	b*	20±3^{r,a}	NA	NA	NA	18±3^{r,b}	NA	NA	NA	19±1^{r,c}	NA	NA	NA
CP1		24±3^{s,a}	26±3^{p,a}	23±2^{p,a}	13±2^{p,b}	18±4^{r,c}	19±3^{o,c}	17±3^{o,c}	14±2^{o,d}	23±3^{s,e}	25±2^{q,e}	15±2^{p,f}	17±1^{o,f}
CP2		19±2^{t,a}	19±2^{q,a}	23±3^{q,b}	17±2^{q,a}	18±2^{r,c}	24±3^{p,d}	21±3^{p,e}	20±3^{p,f}	18±3^{t,g}	18±3^{r,g}	17±2^{q,g}	14±2^{p,h}

[Control samples: Non-treated; CP1, potable water wash + sodium hypochlorite treatment (200 ppm) + gamma irradiation (2 kGy); CP2, potable water wash + sodium hypochlorite treatment (200 ppm) + electron beam irradiation (2 kGy); NA, not available for analysis due to spoilage of sample (control), $^{m\text{ to }t}$ different letters across the column and $^{a\text{ to }h}$ different letters across the row indicates significant difference ($P \leq 0.05$) between the sample means as analyzed by one-way ANOVA]

Organoleptic attributes of combination processed leafy vegetables acceptable

Organoleptic analysis of all the leafy greens was carried out on day 1 of the treatment as well as during the storage period for 15 days at 4-6°C and overall acceptability scores are displayed in Fig. 2. Due to the high microbial load, sensory analysis of control samples was not carried out. Overall acceptability of CP1 as well as CP2 treated spinach samples was 7 (like very well) on the 9-point hedonic scale. The ratings remained unchanged throughout the storage period of 15 days. In case of CP2 treated samples, the overall acceptability score reduced insignificantly ($P \geq 0.05$) during storage on 15 days to 6.5 (Fig. 2). In case of coriander, both CP1 and CP2 treated samples showed overall acceptability scores as 7.5 and no insignificant change was observed during storage. In case of mint, both CP1 and CP2 treated samples showed overall acceptability score of 7.0 that remained unchanged during 15 days of storage. In case of CP2 treated samples, the scores were reduced significantly ($P \leq 0.05$) to 6.0 during the storage of 15 days (Fig. 2). Thus, in case of mint, CP2 treated samples, reduction in overall acceptability during storage was observed. This can be attributed to the higher dose rates of electron beam compared to gamma radiation, resulting in some changes in organoleptic attributes. Nevertheless, the samples were still acceptable till the end of the storage. Fan *et al*.[41] has also showed that when spinach irradiated at doses of 1 or 2 kGy and stored up to 14 days shown acceptability for sensory quality which were evaluated by the expert panel. Thus, as per the organoleptic analysis results, the shelf life of combination treated leafy vegetable samples could be considered up to 15 days at 4-6°C as opposed to the untreated samples. In another study UV-B irradiation treatment to fresh-cut spinach leaves improved quality score and delay of yellowing during 12 days storage at 5°C[43].

Combination process retained phenolics and chlorophyll

Biochemical and functional attributes of leafy greens assayed included total phenolic content, and total chlorophyll content.

Phenolic content

In case of spinach total phenolics content of control samples on day 1 was ~19 mg GAE/g while that of CP1 treated samples was ~23 mg GAE/g (Fig. 3A). During storage, total phenolics decreased slightly but insignificantly ($P \geq 0.05$) till 15th day. In case of CP2 treatment, total phenolics showed significant increase ($P \leq 0.05$) during the storage till 15 days to ~35 mg GAE/g. This could be attributed to the release of

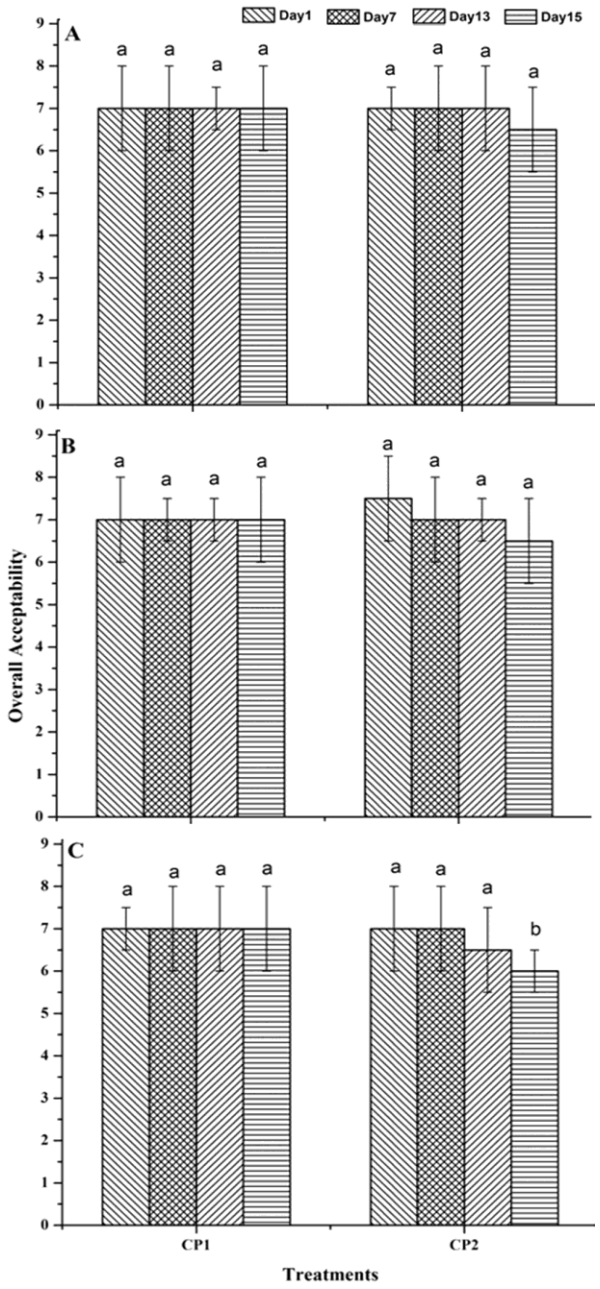

Fig. 2 — Sensory analysis (overall acceptability) of leafy vegetables (A) Spinach; (B) Coriander; and (C) Mint treated with combination process including gamma and EB and stored at 4-6°C. [9 point hedonic scale: 9 = like extremely, 8= like strongly, 7= like very well, 6= like fairly well, 5= like moderately, 4= like slightly, 3= dislike slightly, 2= dislike moderately, 1= dislike extremely. For control samples (non-treated) sensory analysis was not carried out due to contamination with microbial load including presumptive coliforms. CP1: potable water wash + sodium hypochlorite treatment (200 ppm) + gamma irradiation (2 kGy); CP2: potable water wash + sodium hypochlorite treatment (200 ppm) + electron beam irradiation (2 kGy); $^{a\text{-}b}$different letters across the columns indicates significant difference ($P \leq 0.05$) between the sample means as analyzed by one way ANOVA]

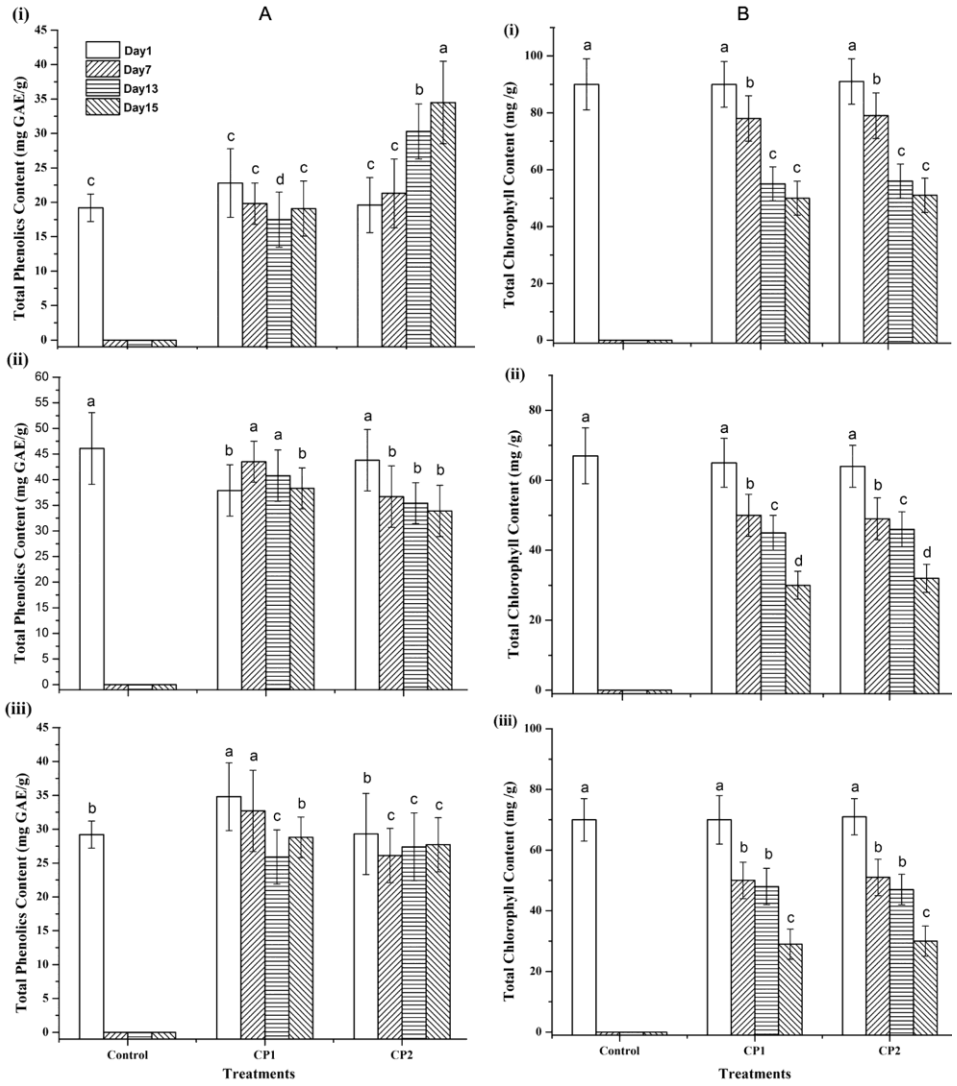

Fig. 3— Biochemical and functional attributes of processed leafy vegetables. (A) Total phenolics content of leafy vegetables (i) Spinach, (ii) Coriander, (iii) Mint; and (B) Total chlorophyll content of leafy vegetables (i) Spinach, (ii) Coriander, (iii) Mint, respectively. [Control samples: non-treated; CP1: potable water wash + sodium hypochlorite treatment (200 ppm) + gamma irradiation (2 kGy); CP2: potable water wash + sodium hypochlorite treatment (200 ppm) + electron beam irradiation (2 kGy); $^{a-d}$ Different letters across the columns and $^{u-x}$ different letters across the rows indicates significant difference ($P \leq 0.05$) between the sample means as analyzed by one way ANOVA]

bound phenols from glycosidic components due to irradiation. Coriander was found to have highest phenolics content among the leafy vegetable studied. In control coriander samples total phenolics were ~46 mg GAE/ g. CP1 treated samples showed total phenolics content of ~38 mg GAE/g at day 1. During storage, there was slight but insignificant increase in the phenolics content to ~44 mg GAE/g on day 7 which then decreased to 38 mg GAE/g on day 15. CP2 treated samples showed total phenolics content of 44 mg GAE/g at day 1 which significantly reduced by ~20% on day 15 (Fig. 3A). Initial phenolics content in control mint samples was ~29 mg GAE/g while in CP1 treated samples it was 35 mg GAE/g. During storage, there was minor but insignificant ($P \geq 0.05$) decrease in the phenolics content till 15 days at 4-6°C. CP2 treated samples showed no change in total phenolics content during storage of 15 days at 4-6°C (Fig. 3A). Similar results have been also obtained by others researchers also they showed that upon irradiation fresh green watercress leaves showed enhancement of antioxidant capacity and content of total phenolics[44].

Chlorophyll content

Chlorophyll content in control as well as CP1 treated spinach sample was 90 mg/g (total chlorophyll) (Fig. 3B). In CP1 treated samples, there was significant

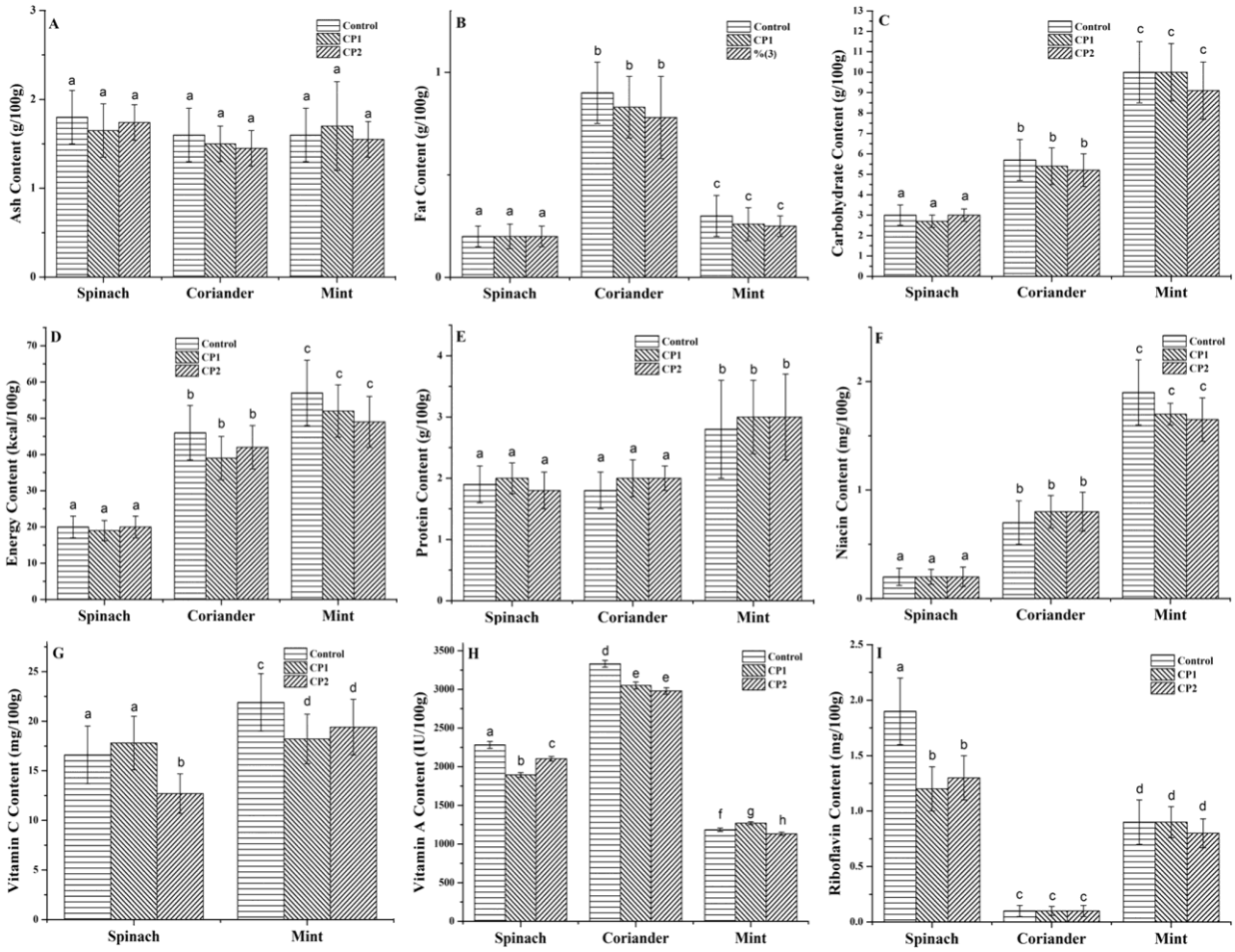

Fig. 4 — Nutritional analysis of fresh control and radiation processed leafy vegetables. (A) Total ash content; and (B) Total fat content; (C) Total carbohydrate content; (D) Total energy content; (E) Total protein content; (F) Total Niacin content; (G) Total vitamin C content; (H) Total vitamin A content; and (I) Total Riboflavin content of spinach, coriander and mint samples, respectively. [Control: Unirradiated samples analyzed on day 1, CP1: Combination (water + sodium hypochlorite (200 ppm) + gamma irradiation 2 kGy)) treated on day 15; CP2: Combination (water + sodium hypochlorite (200 ppm) + electron beam (2 kGy)) treated day 15; All sample analyzed in triplicate and mean results shown. Analysis done under NABL accredited lab. Appropriate positives and blanks have been used. Nutritional attributes of control and irradiated samples (CP1and CP2) were evaluated. $^{j\ to\ r}$ Different letters across the rows indicates significant difference ($P \leq 0.05$) between the sample means as analyzed by one-way ANOVA]

loss ($P \leq 0.05$) of chlorophyll during storage and on 15th day it reduced to 50 mg/g. This could be attributed to the degradation of chlorophyll during storage. In coriander samples, the controls showed total chlorophyll content of 67 mg/g while combination treated (CP1) ones showed 65 mg/g on day 1. Chlorophyll content decreased significantly during storage and it reduced to 30 mg/g on 15th day of storage. Control mint and CP1 treated samples showed chlorophyll content as 70 mg/g (Fig. 3B). In CP1 treated mint samples, there was about 60% reduction in chlorophyll content on 15th day of storage. Reduction in chlorophyll content in leafy greens during storage can be attributed to the degradation of chlorophyll. This is supported by the colorimetric data also (Table 3). In the previous study also researchers observed similar decrease in total chlorophyll content after 3 days of storage in fresh mint sample leaves irradiated at 2 kGy dose of irradiation[42]. Cătunescu *et al*.[45] also observed storage dependent decrease in chlorophyll content in minimally processed parsley leaves during 22 days of storage.

Combination processed samples retained nutritional safety

Ash content in all the leafy greens was in the range of 1.3-1.8 g/100 g (Fig. 4A). There was no significant difference in ash content after the treatments (CP1 and CP2) and between the samples. Total fat content was more (0.9 g/100 g) in coriander and there was no

significant change in fat content after CP1 and CP2 treatments as well as during storage (Fig. 4B). In both spinach and mint, the fat content was in the range of 0.2-0.3 g/100 g and was not altered due to treatment as well as storage (Fig. 4B). Free sugars were absent in all the leafy greens and the carbohydrates content was in the range of 2-10% (Fig. 4C). Among vitamins analyzed, niacin was in the range of 0.2-1.9 mg/100 g (Fig. 4F) while vitamin C was in the range of ~13 to 22 mg/100 g (Fig. 4G). All the leafy vegetables contained high amount of vitamin A that ranged from 1132 to 3331 IU/100 g (Fig. 4H). Significant change ($P \geq 0.05$) has been not occurred in any of the vitamins due to treatment as well as storage. Riboflavin (vit. B2) content in all the leafy greens was very less ranging from 0.1 to 1.9 mg/100 g (Fig. 4I). It showed sensitivity towards radiation and both gamma as well as electron beam irradiated samples showed significant loss of this vitamin. Overall, there was no change in the nutritional parameters except some significant decrease in riboflavin content.

Conclusion

In the current study, leafy agri-produce such as coriander (*Coriandrum sativum* L.), mint (*Mentha spicata* L.) and spinach (S*pinacia oleracea* L.) were found to be significantly burdened with microbes including *E. coli*. Among the presumptive coliforms, 5 isolates were confirmed to be *E. coli* by amplification of *E. coli* specific *uspA* gene in colony PCR. None of the presumptive *Salmonella* isolates showed amplification of *Salmonella* specific *invA* gene in colony PCR. To ensure the safety of such produce and to eliminate the microbial load, a combination of processes including washing with potable water (5 min), sodium hypochlorite (200 ppm, 5 min) and radiation processing at 2 kGy (Gamma or EB) was developed. Studies showed that the microbial load was reduced by about 6 log cycles and the coliform count was below detectable level after the both combination treatments. The treatment did not have any effect on physical, biochemical and functional attributes though there was storage dependent reduction of some of the vitamins like vitamin C (in case of spinach) and niacin (Vit. B3) (in case of mint). Overall, the developed combination treatment was found to be quite efficient in ensuring safety of highly perishable leafy vegetables and also resulted in shelf life extension up to 15 days at 4-6°C. Use of e-beam treatment can make the process considerably cost effective due to high throughput of e-beam processing.

Acknowledgement

Authors thank Dr R Shashidhar, Food Microbiology Section, FTD, BARC for assistance in pathogen screening work.

Conflict of interest

Authors declare no conflict of interests.

References

1 Hee JS & Park JM, The dark side of organic vegetables: interactions of human enteropathogenic bacteria with plants. *Plant Biotechnol Rep*, 13 (2019) 1.

2 Pushpakanth, Periasamy P, Kennedy J, Zachariah & Dananjeyan B, Source tracking of Shiga-like toxin-producing *Escherichia coli* in the fresh vegetable production system of South India. *Ann Microbiol*, 69 (2019) 1.

3 World Health Organization, Food Safety. Available at: https://www.who.int/news-room/fact-sheets/detail/food-safety. Accessed on 27 Jun 2020.

4 Turner K, Moua CN, Hajmeer M, Barnes A & Needham M, Overview of leafy greens–related food safety incidents with a California Link: 1996 to 2016. *J Food Prot*, 82 (2019) 405.

5 Carstens CK, Salazar JK & Darkoh C, Multistate outbreaks of foodborne illness in the United States associated with fresh produce from 2010 to 2017. *Front Microbiol*, 10 (2019) 2667.

6 Centers for Disease Control and Prevention (CDC), Tracking and reporting food-borne disease outbreaks. (2015). Available at http://www.cdc.gov/features/dsfood-borneout-breaks/ Accessed on 03 Feb 2019.

7 Hutchison, M & Moore TP, A study on farm manure applications to agricultural land and an assessment of the risks of pathogen transfer into the food chain, *Technical report, FS 2526* (Min. of Agri. Fisheries and Food, UK), 2019, 26.

8 Soderqvist K, Is your lunch salad safe to eat? Occurrence of bacterial pathogens and potential for pathogen growth in pre-packed ready-to-eat mixed-ingredient salads. *Infect Ecol Epidemiol*, 7 (2017) 1407216.

9 Naik G & Suresh DN, Challenges of creating sustainable agri-retail supply chains. *IIMB Management Review*, 30 (2018) 270.

10 Mritunjay SK & Kumar V, Microbial quality, safety and pathogen detection by using quantitative PCR of raw salad vegetables sold in Dhanbad city, India. *J Food Prot*, 80 (2017) 121.

11 Thapar P & Garcha S, Incidence and characterization of *Pseudomonas* species isolated from spoilt fresh produce. *Indian J Exp Biol*, 55 (2017) 372.

12 Mofoluwaso OJ, David M, Oluwole, Abdallah M & Oladiran F, Occurrence and molecular characterisation of listeria species in some fresh-cut vegetables and environmental samples. *J Adv Microbiol*, 12 (2018) 1.

13 Waghmare R.B & Annapure, US, Integrated effect of radiation processing and modified atmosphere packaging (MAP) on shelf life of fresh fig. *J Food Sci Technol*, 55 (2018) 1993.

14 Ravindran R & Jaiswal AK, Wholesomeness and safety aspects of irradiated foods. *Food Chem*, 285 (2019) 363.

15 Swamy CT, Devraja G, Devaraja TN & Ganesh S, Low dose e-beam irradiation consequence on plant growth promoting properties of lichenized bacteria, *Enterobacter cloacae* and *Providencia rettgeri*. *Indian J Exp Biol*, 57 (2019) 353.

16 Gautam S & Tripathi J, Food processing by Irradiation-An effective technology for food safety and security. *Indian J Exp Biol*, 54 (2016) 700.

17 Food Safety and Standards Authority of India (FSSAI), Atomic Energy (Radiation processing of food and Allied Products) Rules, 2012, *The Gazette of India: Extraordinary [Part III—Sec. 4* (FSSAI, New Delhi), 2016, 8.

18 ASTM International, Standard Practice for Use of a Ceric-Cerous Dosimetry System, *Annual Book of Standard, ASTM* (ASTM International, Switzerland), 2017, 4.

19 Miller A, Batsberg W & Karman W, A new radiochromic thin-film dosimeter system. *Int J Radiat Appl Instrum C Radiat Phys Chem*, 31 (1988) 491.

20 United States food and drug administration, Microbial Analysis. *Bacteriological analytical manual revised* edition, *8th* (US FDA, USA), 2017, 1.

21 Behrsing J, Winkler S, Franz P, & Premier R, Efficacy of chlorine for inactivation of *Escherichia coli* on vegetables. *Postharvest Biol Tech*, 19 (2000) 187.

22 Godambe LP, Bandekar J & Shashidhar R, Species specific PCR based detection of *Escherichia coli* from Indian foods. *3 Biotech*, 7 (2017) 130.

23 Karmi M, Detection of Virulence Gene (*inv-a*) in *Salmonella* isolated from meat and poultry products. *Genetics*, 3 (2013) 7.

24 Onyeoziri IO, Kinnear M & de Kock HL. Relating sensory profiles of canned amaranth (*Amaranthus cruentus*), cleome (*Cleome gynandra*), cowpea (*Vigna unguiculata*) and Swiss chard (*Beta vulgaris*) leaves to consumer acceptance. *J Sci Food Agric*. 98 (2018) 2231.

25 Pathare, P, Opara, U & Fahad AA. Colour Measurement and Analysis in Fresh and Processed Foods: A Review. *Food Bioproc Tech*, 12 (2013) 867.

26 Nunzia C, Maria TL, Margherita P, Mariassunta V & Vincenzo L, A reproducible, rapid and inexpensive Folin-Ciocalteu micro-method in determining phenolics of plant methanol extracts. *Microchem J*, 91 (2009) 107.

27 Arnon DI, Copper enzymes in isolated chloroplasts, poly-phenoxidase in *Beta vulgaris. J Plant Physiol*, 24 (1949) 1.

28 AOAC International, Determination of ash content, Official Method 930.05. (2012b). *Official Methods of Analysis, 19th Edition* (AOAC International, Washington, DC, USA), 2012, 1.

29 Bureau of Indian standard, Bakery product - Method of Analysis, BIS IS 12711 (1989) (reaffirmed: 2005), *Indian Standards* (Bureau of Indian Standards, New Delhi, India), 2005, 3.

30 AOAC, Official Method 923.09. (2012a). Invert sugar in sugars and syrups, In: *Official Methods of Analysis of AOAC International 19th Edn.* (AOAC International, Washington, DC, USA), 2012, 1.

31 Bureau of Indian Standard, Method for determination of protein in foods and feeds, BIS IS 7219 (1973) (reaffirmed 2010) *Indian Standards* (Bureau of Indian Standards, New Delhi, India), 2010, 6.

32 Bureau of Indian Standard, Methods for estimation of nicotinic acid (niacin) in foodstuffs, BIS IS 5400, 1969b (reaffirmed 2010), *Indian Standards* (Bureau of Indian Standards, New Delhi, India), 2010, 11.

33 Bureau of Indian Standard, Method for estimation of vitamin C in foodstuffs, BIS IS 5838-1970 (Reaffirmed 2005), *Indian Standards* (Bureau of Indian Standards, New Delhi, India), 2005, 7.

34 Bureau of Indian Standard, Methods for estimation of carotenes and vitamin A (Retinol) in foodstuff, IS 5886-1970 (Reaffirmed 2010), *Indian Standards* (Bureau of Indian Standards, New Delhi, India), 2010, 5.

35 Bureau of Indian Standard, Methods for estimation of riboflavin (vitamin b2) in, foodstuffs, BIS IS 5399. 1969a, *Indian Standards* (Bureau of Indian Standards, New Delhi, India), 2010, 5.

36 Ssemanda JN, Joosten H, Bagabe MC, Zwietering MH & Reij MW, Reduction of microbial counts during kitchen scale washing and sanitization of salad vegetables. *Food Control*, 85 (2018) 495.

37 Palekar MP, Matthew T, Joseph EM & Castillo A, Reduction of *Salmonella enterica* serotype Poona and background microbiota on fresh-cut cantaloupe by electron beam irradiation. *Int J Food Microbiol*, 202 (2015) 66.

38 Collazo C, Noguera V, Aguiló-Aguayo I, Abadias M, Colás-Medà P, Nicolau I, & Viñas I, Assessing water-assisted UV-C light and its combination with peroxyacetic acid and *Pseudomonas graminis*CPA-7 for the inactivation and inhibition of *Listeria monocytogenes* and *Salmonella enterica* in fresh-cut 'Iceberg' lettuce and baby spinach leaves. *Int J Food Microbiol*, 297 (2019) 11

39 Foley DM, Dufour LA, Rodriguez F, Caporaso & Prakash A, Reduction of *Escherichia coli* O157:H7 in shredded Iceberg lettuce by chlorination and gamma irradiation. *Radiat Phys Chem*, 63 (2002) 391.

40 Olanya OM, Niemira BA & Phillips JG, Effects of gamma irradiation on the survival of *Pseudomonas fluorescens* inoculated on romaine lettuce and baby spinach, *LWT*, 62 (2015) 55.

41 Fan X, Guan W & Sokorai KJB, Quality of fresh-cut Iceberg Lettuce and spinach irradiated at doses up to 4 kGy. *Radiat Phys Chem*, 81 (2012) 1071.

42 Hsu WY, Simonne A, Jitareerat P & Marshall Jr. MR, Low dose irradiation improves microbial quality and shelf life of fresh mint (*Mentha piperita* L.) without compromising visual quality. *J Food Sci*, 75 (2010) M222.

43 Ufuk KM & Rezzan K, Yellowing of fresh-cut spinach (*Spinacia oleracea* L.) leaves delayed by UV-B applications. *Inform Process Agric*, 4 (2017) 214

44 Pinela J, Barros L, Barreira JC, Carvalho AM, Oliveira MB, Santos BC & Ferreira IC, Postharvest changes in the phenolic profile of watercress induced by post-packaging irradiation and modified atmosphere packaging. *Food Chem*, 254 (2018) 70.

45 Cătunescu GM, Muntean M, Marian O, David AP & Rotar AM, Comparative effect of gamma irradiation, drying and freezing on sensory, and hygienic quality of parsley leaves. *LWT*, 115 (2019) 108448.