Se improved indole glucosinolate hydrolysis products content, Se-methylselenocysteine content, antioxidant capacity and potential anti-inflammatory properties of sauerkraut.

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ABSTRACT

Selenium (Se) has a well-known role in prevention of chronic diseases associated with oxidative stress and inflammation. The objective of this study was the production of Se-enriched sauerkraut and to know the effect of selenite addition on indole glucosinolate (GLS) hydrolysis products, vitamin C, Se biotransformation and microbial quality as well as the antioxidant and anti-inflammatory properties of sauerkraut. White cabbage was naturally fermented with 0.3 mg Na_2SeO_3/Kg fresh cabbage (NFSe) or without Se addition (NF). Chemical analyses were performed by LC-PAD, CE and LC-ICP-MS. Antioxidant capacity was determined using the oxygen radical antioxidant capacity (ORAC) method. Anti-inflammatory activity was measured as inhibition of nitric oxide (NO) production in LPS-induced macrophages. Total Se content reached up to 1.29 µg/g dry matter (0.11 µg/g fresh weight) and selenomethylselenocysteine was the major Se form found in NFSe cabbage. Se addition caused a slight reduction of ascorbígeno (6%) and vitamin C (5%) content in sauerkraut ($P \leq 0.05$); however, LAB increased (3%), and the formation of indole-3-carbinol (74%) and indole-3-acetonitrile (13%) were markedly enhanced ($P \leq 0.05$). NFSe cabbage extracts showed higher ($P \leq 0.05$) antioxidant activity (163 µmol Trolox/g d.m.) and anti-inflammatory potency (IC_{50} = 44.01 µg/mL) compared to NF cabbage extracts. Consequently, Se-enriched sauerkraut can be considered as health-promoting food.

KEYWORDS: Fermented cabbage, selenium, glucosinolate breakdown products, antioxidant activity, anti-inflammatory activity

INTRODUCTION

Selenium (Se), as component of selenoamino acids and selenoproteins, is essential in important physiological functions such as redox homeostasis,[1] thyroid hormone metabolism and stimulation of the immune system to increase antibody production.[2] Recently, Se has attracted tremendous interest because of intensive investigation showing the potential of Se to protect against oxidative stress and chronic inflammation, conditions commonly associated to several chronic diseases.[3] Indeed, several human studies suggested that optimal Se status could prevent cancer, cardiovascular disease and type 2 diabetes.[4,5]

Recommended Se dietary intake is not well-standardized among different countries. The recommended dietary allowance (RDA) for Se in USA is 55 μg/day for men and women[6] while the WHO recommends a Se intake of 40 and 30 μg/day for men and women, respectively.[7] Se intake especially in South and Eastern European countries is below RDA[8] and consumption of Se supplements has been the most widespread approach to prevent Se deficiency. Consumption of Se-enriched foods is increasing markedly in recent years as an alternative to increase Se status and promote health of Se-deficient populations.[9] Several authors have reported preparation of Se-enriched foods by means of fermentation processes.[10] Yeasts and lactic acid bacteria have shown its ability to accumulate and transform inorganic Se to organoSe compounds.[10,11] For instance, selenomethionine was found to be the main Se-specie in selenised white wine produced by *Saccharomyces* *cerevisiae* and *Saccharomyces bayanus.*[12] Therefore, Se-enriched fermented foods could become a dietary source of bioavailable and physiologically relevant Se forms.

Sauerkraut, a popular product resulting from the lactic acid fermentation of white cabbage, is a valuable vegetable plant-food due to its nutritional and health-promoting properties, mainly attributed to its high content of antioxidant compounds such as vitamin C and phenolic compounds[13,14] which prevent cell damage caused by free radicals. Besides that, sauerkraut presents high levels of glucosinolate (GLS) breakdown products[15] among them, indole-3-carbinol (I3C), indole-3-acetonitrile (I3ACN) and ascorbigen (ABG) are the most abundant.[14,15,16] The GLS-breakdown products have been linked with a reduction of cancer risk by inhibiting phase I enzymes, involved in carcinogen activation and inducting phase II enzymes, involved in the detoxification of xenobiotics.[17,18] These compounds also inhibit tumor cell growth and stimulate apoptosis.[19] Different authors described the relationship between inflammation and cancer.[20]

There is no literature information on the effect of the Se addition during cabbage fermentation on the bioactive compounds and biological properties of sauerkraut. Therefore, the objective of this study was the production of Se-enriched sauerkraut and to know the effect of selenite addition on indole GLS hydrolysis products, vitamin C, Se biotransformation and microbial quality as well as the antioxidant capacity and anti-inflammatory properties of sauerkraut.

MATERIAL AND METHODS

Plant material. White cabbages (*Brassica oleracea* L. var. *capitata* cv. Megaton) grown in the North region of Spain (La Rioja) during winter season 2008 were selected among five Spanish cultivars, based on their glucobrassicin content.[21] Fresh cabbages were provided by Bejo Iberica S. L. (Madrid, Spain) and fermented immediately upon reception.

Preparation of Se-enriched sauerkraut. Cabbage heads were prepared by removing the outer leaves and their central core. The edible part of cabbages was then shredded into about 2 mm thick strips using a shredder (Moka Express, Barcelona, Spain). Subsequently, 0.5% NaCl and 0.3 mg of sodium selenite/Kg of fresh cabbage (1.6 mg Se/Kg of dry matter) were added to achieve Se RDA and a high selenite biotransformation in selenoaminoacids. Shredded cabbage and brine were mixed thoroughly, transferred to sterile polyethylene vessels (8 L) and tightly pressed to exclude air. Fermentations were performed spontaneously by the indigenous microbiota present on raw cabbage (NFSe). Sauerkraut without addition of Se were also prepared and considered as control (NF). Fermentations were carried out in 3 batches (4 Kg per batch) at room temperature (22-25 ºC) for 7 days. On the third day, cabbage was pricked to remove releasing gases. Raw, NF and NFSe cabbages were freeze-dried, milled and stored at -20 ºC under vacuum until their analysis.

Determination of pH during fermentation. Brine from each fermentation batch (2 mL) was collected at 0, 3 and 7 days of fermentation and their pH was measured in a pH meter Basic 20 (Crison, Barcelona, Spain).

Analysis of indole GLS-derived compounds. The content of ABG, I3C and I3ACN was determined by HPLC-PAD in raw and fermented cabbage as in Peñas et al.[14]

Determination of vitamin C. The quantification of vitamin C content in raw and fermented cabbages was performed by capillary electrophoresis (CE) as in Frias et al.[22] CE analysis were performed in a P/ACE system 2050 (Beckman Instruments, Fullerton, CA, USA) and UV detector at 254 nm. Separation was done using a 47 cm x 75 μm i.d. fused silica capillary TSP075375 column (Composite Metal Services LTD, Worcester, UK) at room temperature.

Oxygen Radical Antioxidant Capacity (ORAC). ORAC was determined in aqueous extracts from raw and fermented cabbage extracts. Extracts were obtained by suspension of 1 g of freeze-dried sample in 10 mL of phosphate buffer (PBS, pH 7.4) and stirring for 1 h at room temperature. Homogenates were filtered using Whatman No.1 filter paper. The ORAC assay was determined as described by Dávalos et al.[23] Results were expressed as µmol Trolox equivalents (TE) per gram of dry matter (µmol TE/g d.m.).

Determination of Se-derived compounds. Total selenium concentration was determined in raw and fermented cabbages by ICP-MS (Thermo-X Series) after acid digestion in an analytical microwave oven (CEM MSP 1000, Matheus, NC) by following conditions described previously.[10,11] Selenium speciation was carried out by HPLC-ICP-MS previous enzymatic probe sonication.[10,11] Enzymatic hydrolysis was performed by 2 minutes of sonication (Sonoplus ultrasonic homogenizer Bandenlin, Germany) after addition of 20 mg of Protease XIV (Sigma-Aldrich, Steinheim, Germany) and 3 mL of Milli-Q water to 100 mg of dried sample. The obtained extracts were centrifuged at at 15,557 *x g* for 30 min (4 ºC) using 10 KDa cut-off filters (Millipore, USA). The ICPMS instrument was coupled with a Hamilton PRP-X100 (250 x 4.1 mm, 10 µm) chromatographic column. The mobile phase was 10 mM ammonium citrate, pH 5.0, in 2% methanol as mobile phase at a flow rate of 1mL/min**.** Identification and quantification of selenium species was done by retention time and spiking experiments. Standard stock solutions of 1000 mg/L of selenomethionine (SeMet), selenomethylselenocysteine (SeMeSeCys) and selenocystine ($SeCys_2$) (Sigma) were prepared in ultra-pure Milli-Q water (Millipore, MA, USA), and 3% hydrochloric acid was added for better dissolution of $SeCys_2$ and SeMeSeCys. Inorganic selenium solutions were prepared by dissolving sodium selenite (Na_2SeO_3) and selenate (Na_2SeO_4) (Merck) in Milli-Q water.

Preparation of extracts for cell treatment. Extracts were prepared by homogenization of 500 mg freeze-dried sample in 20 mL acetone:water solution (1/1) using an Ultra Turrax homogenizer T-25 Digital (Ika Werke GMBH & Co., Staufen, Germany), and centrifugation for 7 min at 3,024 *x g* and 5 ℃. Supernatant was collected, and the pellet was extracted twice with 10 mL of acetone. Further, supernatants were combined, filtered using Whatman No. 1 paper and concentrated to 7 mL final volume. The concentrate was extracted twice with 15 mL of ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated under vacuum to dryness. Finally, the residue was dissolved in 0.1% dimethylsulfoxide (DMSO) (Sigma).

Cell viability assay. Macrophages cell line RAW 264.7 (ATCC, Manassas, VA, USA) were cultured in Dubelcco´s modified Eagle Medium (DMEM; from ATCC) containing 1% penicillin/streptomycin (Sigma), and 10% fetal bovine serum (ATCC) at 37 ºC in 5% CO_2 atmosphere. The cell proliferation assay was conducted using the CellTiter 96 Aqueous One Solution Proliferation assay kit using the novel tetrazolium compound,3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), and an electron coupling reagent, phenazine ethosulfate (PES) (Promega Biotech Iberica, Madrid, Spain). Briefly, 5 x 10^4 cells/well were seeded in a 96-well plate and the total volume was adjusted to 200 μL with DMEM. The cells were allowed to grow for 24 h at 37 ºC in 5% CO_2. After 24 h incubation, they were treated with different concentrations of raw and fermented cabbage extracts (0-150 μg/mL), ABG (0-1000 μM) and SeMeSeCys (0-10 μM) for 24 h. After treatment, DMEM was replaced by 100 μL fresh medium and 20 μL MTS/PES was added to each well. The plate was incubated for 2 h at 37 ºC and the absorbance read at 490 nm. The percentage of viable cells was calculated with respect to cells treated with vehicle (0.1% DMSO) as follows: $A_{treatment\ 490\ nm}/A_{control\ 490\ nm} * 100$ = % cell viability

Measurement of nitric oxide (NO). Approximately 5 x 10^4 cells/well were seeded in a 96-well plate and allowed to grow to its 80-

90% confluency. The cells were treated with 1 μg/mL lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (Sigma) with or without different concentrations of raw and fermented cabbage extracts (0-150 μg/mL), ABG (0-1000 μM) and SeMeSeCys (0-10 μM) for 24 h. After this treatment, medium was collected and NO production analyzed. Nitrite accumulation, and indicator of NO synthesis, was measured in the culture medium by Griess reaction.[24] Briefly, 100 μL of DMEM were plated in 96-well plate and an equal amount of Griess reagent constituted by 1% (w/v) sulfanilamide and 0.1% (w/v) N-1-(naphthyl)ethylenediamine-diHCl in 2.5% (v/v) H_3PO_4, was added. The plate was incubated for 5 min and the absorbance measured at 550 nm in a microplate reader (Biotek, Winooski, VT, USA). The amount of NO was calculated using a sodium nitrite standard curve. Potency was determined by dose–response curves in which the range of concentrations was distributed in a logarithmic scale and the IC_{50} values were calculated using non-linear regression sigmoidal curve fit functions in GraphPad Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA).

Microbiological analysis. Microbiological analyses were performed on raw and fermented cabbage as described by Peñas et al.[25] Briefly, five grams of each sample were aseptically diluted in buffered peptone water (Scharlau Chemie, Spain) in a sterile Stomacher bag and homogenised for 1 min in a Stomacher laboratory blender (IUL Masticator, Barcelona, Spain). Further serial dilutions were made for plating. The pour plate technique was employed to determine the microbial counts. Total aerobic and anaerobic mesophilic bacteria were enumerated on Tryptone Soya Agar (TSA) after incubation in aerobic and anaerobic conditions, respectively, at 30 ºC for 72 h; total and faecal coliforms on Violet Red Bile Agar (VRBA) containing lactose as carbohydrate source, after incubation at 37 ºC and 44 ºC, respectively, for 24 h; moulds and yeasts on Sabouraud-Chloramphenicol Agar, after incubation at 23 ºC for 96 h; and LAB on MRS Agar after incubation in anaerobic conditions at 30 ºC for 24-48 h.

Statistical analysis. Data were expressed as means of three experiments. Results were compared by one-way analysis of variance (ANOVA) using the least significant differences ($P \leq 0.05$) (Statgraphic 5.0 software, Statistical Graphics Corporation, Rockville, MD, USA).

RESULTS AND DISCUSSION

Evolution of pH during cabbage fermentation. Figure 1 shows pH evolution during cabbage fermentation in the presence (NFSe) or absence (NF) of selenite solution. Measurement of pH can be considered as an indicator of the success of fermentation processes.[26] Raw cabbage pH (6.2) was found similar to the one observed previously by our group in white cabbage cv. Bronco.[14] A rapid pH decrease took place up to 3 days of fermentation as a result of organic acid production by indigenous LAB in NF (pH 3.6) and NFSe (pH 3.7) cabbages, and it did not change up to 7 days of fermentation (Figure 1). Our results are consistent with previous studies in cabbages submitted to natural or induced fermentation using LAB starter cultures.[14,27,28] Moreover, selenite addition had no significant influence ($P \leq 0.05$) on pH evolution during sauerkraut production (Figure 1). Therefore, Se addition seemed to have no adverse effect on LAB fermentative metabolism during sauerkraut production.

Effect of Se-enrichment on the content of indole GLS breakdown products in natural fermented cabbage. Raw cabbage showed small concentrations of indole GLS hydrolysis products such as ABG (~16 µmol/100g d.m.), I3C (0.10 µmol/100g d.m.) and I3ACN (0.24 µmol/100g d.m.) since cabbage was shredded immediately before analysis (Table 1). ABG concentration found in raw cabbage was within the range reported in previous studies.[14,16] However, no literature data has been found about I3C and I3ACN content in raw white cabbage.

During natural fermentation, the content of indole GLS hydrolysis products markedly increased ($P \leq 0.05$), being ABG (218.2 µmol/100g d.m.) the major indole GLS-derived compound found in NF cabbage (Table 1), followed by I3C (9.6 µmol/100g d.m.), and I3ACN (2.7 µmol/100g d.m.). The content of indole GLS-derived products in NF cabbage differed from previous studies[14-16,29] which could be attributed to differences in glucobrassicin concentration and myrosinase activity as they depend on genetic and environmental factors that vary among growing locations and year of cultivation.[21,30]

NFSe cabbages showed significantly lower ABG content but higher I3C and I3ACN concentrations ($P \leq 0.05$), compared to NF cabbages (Table 1). No previous studies have been found on the effect of Se on the formation of GLS hydrolysis products during cabbage fermentation. Indole GLS breakdown products are considered anticarcinogenic agents that are associated to lower risk of breast[31] and colon cancer.[32] Furthermore, ABG exert important immunomodulating actions[33] while I3C promote cell cycle arrest in endometrial[34] and prostate tumors.[35]

Effect of Se-enrichment on ascorbic acid content of natural fermented cabbage. As it can be observed in Table 2, raw cabbage presented a high vitamin C content (~329 mg/100 g d.m.). These results are in agreement with those previously reported for different white cabbage cultivars.[14,16,21] Ascorbic acid content decreased more than 25% in NF cabbage compared to raw cabbage. This reduction could be explained by ABG formation which results from the hydrolysis of glucobrassicin by myrosinase enzyme and the further reaction with L-ascorbic acid at low pH,[36] and the oxidation of ascorbic acid during cabbage fermentation. The level of vitamin C found in the present work was consistent with our previous studies.[14,16] Fermentation in the presence of Se caused a small but significant ($P \leq 0.05$) decrease of vitamin C in NFSe cabbage. No information was found about the effect of Se on ascorbic acid content in sauerkraut.

Biotransformation of selenite during natural fermentation of white cabbage. Table 2 collects the content of Se compounds in raw, NF and NFSe cabbages. Total Se in raw and NF cabbage was 0.04 and 0.07 µg/g d.m. respectively, and Se species concentration were lower than the quantification limit. Selenite addition into cabbage caused a 19.5-fold increase of total Se concentration up to 1.29 µg/g d.m. (equivalent to 11.62 µg/100 g f.w.) and Se-methylselenocysteine (SeMeSeCys) (0.74 µg/g d.m.) was the major Se specie found in Se-enriched sauerkraut. Therefore, the consumption of a serving of 150 g f.w. of Se-enriched sauerkraut would contribute to cover a relevant percentage of Se RDA (43.6% for men and 58.1% for women). With regard to safety, upper limit of the safe adult population mean intake of selenium have been established in 400 μg Se/d.[7] A Se-enriched sauerkraut serving of 150 g f.w. contains 17.43 μg Se, levels that are in the safe range of Se intake.

After enzymatic hydrolysis using protease XIV, samples were ultrafiltrated through 10 kDa cutoff membranes. The Se recovery in ultrafiltrated cabbage hydrolysates was 90±3%. The same extraction procedure was applied without protease (aqueous extraction) providing recovery values of 60±3%. These results suggested that most of Se in samples was not bound to proteins. Figure 2 shows the chromatographic profiles of selenium species standards (Figure 2A) and the raw, NF, NFSe cabbage hydrolysates (Figure 2B). It is noteworthy the production of SeMeSeCys during fermentation in the presence of selenite. SeMeSeCys was also the main Se-compound found when samples were treated by aqueous extraction. SeMeSeCys is a non-proteinogenic selenoamino acid which is metabolized by lyase to methylselenol *in vivo*.[37] This fact is very important because there is evidence that ability of endogenous production of monomethylated selenium is a critical factor in Se chemoprotection. Recently, Cuello et al.[38] have reported that SeMeSeCys protects human hepatoma cells against oxidative stress.

The ability of some microorganisms to biotransform inorganic Se has been widely reported in the literature, especially *Sacharomyces* *cerevisiae.* Yeasts are being used as Se supplement because its ability to accumulate and biotransform high concentrations of inorganic selenium (3000 μg/g) mainly into selenomethionine (SeMet).[11,12] Thus, several authors have reported preparation of selenized foodstuffs mediated by a fermentation process.[10,12] Alzate et al.,[10] compared the different Se species that are produced when lactic fermentation in presence of two different types of microorganisms, LAB (*Lactobacillus*) and yeast (*Saccharomyces*) take place to produce yogurt and kefir, respectively. Se organic species formed depended on the type of microorganism involved in the fermentation process, being $SeCys_2$ and SeMeSeCys the main Se species generated by *Lactobacillus* and SeMet by *Saccharomyces*. These results are in agreement with those reported in the present work, and others previously published supporting the different behaviour of microorganisms *Lactobacillus*[39,40] and *Sacharomyces* when exposed to inorganic Se.[11]

Results illustrated in Table 2 evidence that up to 50% of the total Se content found in Se-enriched sauerkraut was identified as SeMeSeCys. This Se specie was not found in raw cabbage which again suggests that its production is derived from the action of the microorganisms during the fermentation process in presence of inorganic Se.

Effect of Se-enrichment on the antioxidant capacity of natural fermented cabbage. The antioxidant activity of raw cabbage was ~75 μmol TE/g d.m. and natural fermentation (NF) led to a sharp increase (131.5 μmol TE/g d.m.) (Table 3). This effect was also observed by other authors in spontaneously fermented cabbage.[41] These results can be attributed to the formation of GLS-derivatives during fermentation that act as direct antioxidant compounds.[42] GLS hydrolysis products may enhance antioxidant potential indirectly by the action of antioxidant responsive element which induced gene expression of phase II enzymes in murine models.[43] Other factors also contribute to the enhanced antioxidant activity found in sauerkraut compared to raw cabbage. According to Reyes et al.,[44] the antioxidant activity of cabbage increases during wounding, effect that takes place after shredded during cabbage fermentation. Additionally, LAB may also affect the antioxidant activity exhibited by the fermented cabbage, as has been previously suggested by Kusznierewicz et al.[45]

Fermentation in the presence of Se significantly improved ($P \leq 0.05$) antioxidant activity in sauerkraut (Table 3). The highest antioxidant activity found in NFSe cabbage compared to NF cabbage may be related to the biotransformation of selenite in Se-organic species which are able to scavenge free radicals.[46]

Effect of Se-enrichment on microbiological quality of sauerkraut. Microbial counts of raw, NF and NFSe cabbage are shown in Table 4. The dominating microbial population of raw cabbage was aerobic mesophilic bacteria (~5 cfu/g), followed by anaerobic bacteria (~4 cfu/g), LAB (~2 cfu/g) and total coliforms (~1 cfu/g). Faecal coliforms, moulds and yeasts were not detected in raw cabbage. These results are consistent with those recently reported for other *Brassica* vegetables[47] and white cabbage cv. Bronco.[25]

Fermentation caused a significant ($P \leq 0.05$) increase of aerobic and anaerobic mesophilic bacteria (6.8 cfu/g f.w.) as well as LAB (6.7 cfu/g f.w.) in NF cabbage; however, microbial counts of faecal coliforms, moulds and yeasts did not change after fermentation (Table 4). LAB grew in greater extent than other microbial populations, due to the favourable ecological conditions (acidic pH and low oxygen concentration) that take place during cabbage fermentation, enhancing their multiplication and inhibiting the growth of other microorganisms such as aerobic mesophilic bacteria. Slightly lower counts of aerobic mesophilic bateria and LAB were found in sauerkraut obtained by spontaneous fermentation from white cabbage cv. Bronco,[25] probably due to differences in endogenous microflora.

The addition of Se during cabbage fermentation caused significantly ($P \leq 0.05$) lower aerobic and anaerobic mesophilic bacteria counts and significantly ($P \leq 0.05$) higher LAB counts. These findings suggest that Se-enrichment enhanced the growth of LAB and, consequently, other microbial populations decreased most likely due to a competition phenomenon. Recently, Molan et al.[48] reported that inorganic forms of Se (selenate and selenite) exert a prebiotic effect as evidenced by their ability to promote the growth of *Lactobacillus rhamnosus* and *Bifidobacterium breve in vitro*. Furthermore, these authors found that Se-enriched green tea enhanced lactobacilli growth compared to conventional green tea.

Effect of raw and fermented cabbage extracts on inflammatory response of LPS-induced RAW 264.7 macrophages.

The cytotoxicity of raw and NF and NFSe cabbage extracts in LPS-induced macrophages was evaluated at a range 0-150 μg extract/mL using MTS reduction assay after 24 h incubation. Macrophages exhibited a survival percentage > 87% when treated with raw and fermented cabbage extracts at concentrations ≤ 150 μg/mL. Therefore, these results indicated that range of concentrations used in this study to treat the cells did not exert any cytotoxic effect. In activated macrophages, NO production noticeably increased in response to LPS as shown in Figure 3. Excessive production of NO in macrophages represent a potentially toxic effect, which if not counteracted, causes the onset or/and progression of many disease pathologies.[49] Therefore, the effect of raw, NF and NFSe cabbage extracts on inhibition of NO production was examined in order to study their potential anti-inflammatory effect. Raw, NF and NFSe cabbage extracts induced a significant ($P \leq 0.05$) dose-dependent suppression of NO production (Figure 3A). Table 3 shows the calculated concentrations of raw, NF and NFSe cabbage extracts that resulted in 50% inhibition of NO production in LPS-induced macrophages (IC_{50}). NF cabbage extract exhibited higher ($P \leq 0.05$) potency (IC_{50} = 83.96 μg/mL) compared to raw cabbage extract (IC_{50} = 167.93 μg/mL). This effect could be due to formation of bioactive compounds such as GLS breakdown products exhibiting anti-inflammatory properties during sauerkraut manufacture. ABG was the major GLS hydrolysis compound in NF cabbage (Table 1), therefore, we further investigated the potential anti-inflammatory activity of synthetic ABG in LPS-induced macrophages. The cytotoxicity of ABG was firstly examined after 24 h incubation.

Macrophages exhibited a viability > 92% which indicated that ABG did not induce any cytotoxic effect at concentrations ≤ 1000 μM. ABG was able to significantly (P ≤ 0.05) reduce NO production in a dose-dependent manner (Figure 3B); however, ABG was a weak inhibitor of NO production in LPS-activated macrophages (IC_{50} = 970.54 μM). These results suggest that ABG have a small contribution (<10%) to the NO production inhibitory potency indicating that other bioactive compounds would be responsible for the observed potential anti-inflammatory activity of fermented cabbage extracts. A small body of literature suggests that I3C and sulforaphane (SF) may protect against inflammation, inhibiting cytokine production (TNF-α, IL-1, IL-6, IL-8) and expression of proinflammatory enzymes such inducible nitric oxide synthase (iNOS).[50,51] Therefore, it will be interesting to see if ABG, SF and I3C have synergistic effects in fighting inflammation.

Selenite addition (1.6 mg Se/Kg d.m.) markedly improved the NO production inhibitory potency of fermented cabbage (IC_{50} = 44 μg extract/mL). To further confirm the role of Se in the modulation of inflammatory response we tested the effect of SeMeSeCys, the major Se specie found in NFSe cabbage, on NO production in LPS-activated macrophages. Treatment with SeMeSeCys (concentration range 0-10 μM) caused a dose-dependent inhibition of NO production in LPS-activated macrophages (Figure 3C) and showed no cytotoxicity at concentrations ≤ 10 μM. SeMeSeCys potency to inhibit 50% NO production in LPS-activated macrophages (IC_{50}) was 25.20 μM (equivalent to 10.93 μM of Se). This level of Se represents a serumachievable concentration of total Se and is less than the lower 95% confidence limit of the non-observed adverse effect level (NOAEL).[52] Taking into consideration SeMeSeCys content as well as NO production inhibitory potency (IC_{50}) of Se-enriched sauerkraut it was estimated that this compound have a relative contribution of 38% to the IC_{50} value. These results suggest that SeMeSeCys could be acting synergistically with other anti-inflammatory compounds present in Se-enriched fermented cabbage. Our results agree with reports demonstrating the effect of Se on attenuation of proinflammatory response in various cell lines.[53] Several studies have been consistent showing the ability of Se in the regulation of expression of the proinflammatory enzymes iNOS and cycloxigenase 2 (COX-2) through inactivation of the nuclear transcription factor B (NF-κB).[54,55] Therefore, downregulation of proinflammatory gene expression by Se explains the inhibition of NO production in LPS-induced macrophages showed in the present study.

In conclusion, the addition of sodium selenite during natural cabbage fermentation enhanced the formation of some GLS breakdown compounds that exhibits health promoting properties, increased the antioxidant and potential anti-inflammatory activities of sauerkraut. On the other hand, SeMeSeCys was the primary selenocompound observed in the resulting Se-enriched sauerkraut. Consequently, the consumption of Se-enriched sauerkraut will contribute the Se dietary intake and it can be considered as a good source of health-promoting compounds.

REFERENCES

(1) Behne, D.; Alber, D.; Kyriakopoulos, A. Long-term selenium supplementation of humans: Selenium status and relationships between selenium concentrations in skeletal muscle and indicador materials. *J.Trace Elem. Med. Biol.* **2010**, *24*, 99–105.

(2) Hartikainen, H. Biogeochemistry of selenium and its impact on food chain quality and human health. *J. Trace Elem. Med. Biol.* **2005**, *18*, 309–318.

(3) Navarro-Alarcon, M.; Cabrera-Vique, C. Selenium in food and the human body: A review. *Sci. Total Environ.* **2008**, *400*, 115-141.

(4) Reid, M. E.; Duffield-Lillico, A. J.; Slate, E.; Natarajan, N.; Turnbull, B.; Jacobs, E.; Combs, G. F.; Clark, L. C.; Marshall, J. R. The nutritional prevention of cancer: 400 mcg per day selenium treatment. *Nutr. Cancer* **2008**, *60*, 155-163.

(5) Stranges, P.; Nava-Acien, A.; Rayman, M.P.; Guallar, E. Selenium status and cardiometabolic health: State of the evidence. *Nutr. Metabol. Cardiov. Dis.* **2010**, *20*, 754-760.

(6) Dietary reference intakes (DRI). National Research Council. Washington: National Academy Press. **2000**. pp. 284–319.

(7) WHO/FAO/IAEA. Trace elements in human nutrition and Health. Geneva: World Health Organization, 1996.

(8) Rayman, M. P. Food-chain selenium and human health: emphasis on intake. *Br. J. Nutr.* **2008**, *100*, 254-268.

(9) Hu, Y.; McIntosh, G. H.; Le Leu, R. K.; Young, G. P. Selenium-enriched milk proteins and selenium yeast affect selenoprotein activity

and expression differently in mouse colon. *Br. J. Nutr*. **2010**, *104*, 17-23.

(10) Alzate, A.; Fernández-Fernández, A.; Pérez-Conde, C.; Gutiérrez, A. M.; Cámara, C. Comparison of biotransformation of inorganic selenium by *Lactobacillus* and *Saccharomyces* in lactic fermentation process of yogurt and kerfir. *J. Agric. Food Chem.* **2008**, *56*, 8728-8736.

(11) Ponce de León, C. A.; Bayón. M. M.; Paquín, C.; Caruso, A. Selenium incorporation into *Saccharomyces cerevisiae* cells: a study of different incorporation methods. *J. Appl. Microbiol.* **2002**, *92*, 602-610.

(12) Pérez-Corona M. T.; Sánchez-Martínez, M.; Valderrama, M. J.; Rodríguez, M. E.; Cámara C.; Madrid Y. Se Biotransformation by *Saccharomyces cerevisiae* and *S. bayanus* during white wine manufacture: Lab-scale experiments. *Food Chem.* **2011**, *124*, 1050-1055.

(13) Harmaum, B.; Hubbermann, E. M.; Zhu, Z.; Schwarz, K. Impact of fermentation on phenolic compounds in leaves of pak choi (*Brassica* L. ssp. *chinesis* var. *communis*) and chinese leaf mustard (*Brassica juncea coss*). J. Agric. Food Chem. 2008, 56, 148-157.

(14) Peñas, E.; Frias, J.; Sidro, B.; Vidal-Valverde, C. Chemical evaluation and sensory quality of sauerkrauts obtained by natural and induced fermentations at different NaCl levels from *Brassica oleracea* var. *capitata* cv. Bronco grown in Eastern Spain. Effect of storage. *J. Agric. Food Chem.* **2010**, *58*, 3549-3557.

(15) Ciska, E.; Pathak, D. Glucosinolate derivatives in stored fermented cabbage. *J. Agric. Food Chem.* **2004**, *52*, 7938-7943.

(16) Martinez-Villaluenga, C.; Peñas, E.; Frias, J.; Ciska, E.; Honke, J.; Piskula, M. K.; Kozlowska, H.; Vidal-Valverde, C. Influence of fermentation conditions on glucosinolates, ascorbigen, and ascorbic acid content in white cabbage (*Brassica oleracea* var. *capitata* cv. Taler) cultivated in different seasons. *J. Food Sci.* **2009**, *74*, C62-C67.

(17) Das, S.; Tuagi, A. K.; Haur, H. Cancer modulation by glucosinolates: a review. *Curr. Sci*. **2000**, *79*, 1665–1671.

(18) Mithen, R. F. Glucosinolates and their degradation products. *Adv. Bot. Res*. **2001**, *35*, 213–262.

(19) Johnson, I. T. Glucosinolates in the human diet. Bioavailability and implications for health. *Phytochem. Rev.* **2002**, *1*, 183–188.

(20) Grivennikov, S. I., Karin, M. Inflammation and oncogenesis: a vicious connection. *Curr. Opin. Genet. Dev.* **2010**, *20*, 65–71.

(21) Peñas, E.; Frias, J.; Martinez-Villaluenga, C.; Vidal-Valverde, C. Bioactive compounds, myrosinase activity and antioxidant capacity of white cabbages grown in different locations of Spain. *J. Agric. Food Chem*. **2011** (submitted).

(22) Frias, J.; Miranda, L. M.; Doblado, R.; Vidal-Valverde, C. Effect of germination and fermentation on the antioxidant vitamin content and antioxidant capacity of *Lupinus albus* L. var. *multolupa*. *Food Chem.* **2005**, *92*, 211-220.

(23) Davalos, A.; Gomez-Cordoves, C.; Bartolome, B. Extending applicability of the oxygen radical absorbance capacity (ORAC-Fluorescein) assay. *J. Agric. Food Chem*. **2004**, *52*, 48-54.

(24) Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. *Anal. Biochem*. **1982**, *26*, 131-138.

(25) Peñas, E.; Frias, J.; Gomez, R.; Vidal-Valverde, C. High hydrostatic pressure can improve the microbial quality of sauerkraut during storage. *Food Control* **2010**, *21*, 524-528.

(26) Johanningsmeier, S.; McFeeters, R. F.; Fleming, H. P.; Thompson, R. L. Effects of *Leuconostoc mesenteroides* starter culture on fermentation of cabbage with reduced salt concentrations. *J. Food Sci*. **2007**, *72*, 166-172.

(27) Viander, B.; Maki, M.; Palva, A. Impact of low salt concentration, salt quality on natural large-scale sauerkraut fermentation. *Food Microbiol*. **2003**, *20*, 391-395.

(28) Kohajdová, Z.; Karovicová, J.; Greifová, M. Lactic acid fermentation of some vegetable juices. *J. Food Nutr*. Res. **2006**, *45*, 115-119.

(29) Ciska, E.; Verkerk, R.; Honke, J. Effect of boiling on the content of ascorbigen, indol-3-carbinol, indol-3-acetonitrile, and 3,3´-diindolylmethane in fermented cabbage. *J. Agric. Food Chem*. **2009**, *57*, 2334-2338.

(30) Rosa, E. A. S.; Hesney, R. K.; Fenwick, G. R.; Portas, C. Glucosinolates in crop plants. *Hort. Rev*. **1997**,*19*, 99-215

(31) Fowke, J. H.; Chung, F. L.; Jin, F.; Qi, D.; Cai, Q.; Conoway, C.; et al. Urinary isothiocyanate levels, brassica, and human breast cancer. *Cancer Res*. **2003**, *63*, 3980-3986.

(32) Bonnesen, C.; Eggleston, I. M.; Hayes, J. D. Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and conver protection against DNA damage in human colon cell lines. *Cancer Res*. **2001**, *61*, 6120-6130.

(33) Preobrazhenskaya, M. N.; Bukhman, V. M.; Korolev, A. M.; Efimov, S. A. Ascorbigen and other indole-derived compounds from Brassica vegetables and their analogs as anticarcinogenic and immunomodulating agents. *Pharmacol. Ther*. **1993**, *60*, 301-313.

(34) Kojima, T.; Tanaka, T.; Mori, H. Chemoprevention of spontaneous endometrial cancer on female Donryku rats by dietary indole-3-carbinol. *Cancer Res.* **1994**, *5*, 1446-1449.

(35) Souli, E.; Machluf, M.; Morgenstern, A.; Sabo, E.; Yannai, S. Indole-3-carbinol (I3C) exhibits inhibitory and preventive effects on prostate tumours in mice. *Food Chem. Toxicol.* **2008**, *46*, 815-828.

(36) Hrncirik, K.; Valusek, J.; Velisek, J. A study on the formation and stability of ascorbigen in an aqueous system. *Food Chem.* **1998**, *63*, 349–356.

(37) Ganther, H. E.; Lawrence, J. R. Chemical transformations of selenium in living organisms. Improved forms of selenium for cancer prevention. *Tetrahedron*, **1997**, *53*, 12299-12310.

(38) Cuello, S.; Ramos, S.; Mateos R.; Martin, M. A.; Madrid Y.; Cámara C.; Bravo, L; Goya, L. Selenium methylselonocysteine

protects human hematoma HepG2 cells against oxidative stress induced by tert-butyl 6 hydroperoxide. *Anal. Bioanal. Chem.* **2007**, *389*, 2167-2178.

(39) Andreoni, V.; Moro-Luischi, M.; Cavalca, L.; Erba, D.; Ciapellano, S. Selenite tolerance and accumulation in *Lactobacillus* species. *Ann. Microbiol.* **2000**, *50*, 77-88.

(40) Calome, M.R.; Vand den Branden, K.; Vanden Berghe, D. A. Selenium and *Lactobacillus* species. *J. Appl. Bacteriol.* **1995**, *79*, 331-340.

(41) Bartoszek, A.; Forc, A.; & Grzeskowiak, J. Antioxidative properties of some vegetable products traditional for diets in Central Europe – Short Report. *Polish J. Food Nutr. Sci.* **2002**, *11*, 67–70.

(42) Barillari, J.; Canistro, D.; Paolini, M.; Ferroni, F.; Pedulli, G. F.; Iori, R.; Valgimigli, L. Direct antioxidant activity of purified glucoerucin, the dietary secondary metabolite contained in rocket (*Eruca sativa* Mill.) seeds and sprouts. *J. Agric. Food Chem.* **2005**, *53*, 2475–2482.

(43) Vig, A. P.; Rampal, G.; Thind, T. S.; Arora, S. Bio-protective effects of glucosinolates – A review. *LWT-Food Sci. Technol.* **2009**, *42*, 1561–1572.

(44) Reyes, L. F.; Villarreal, J. E.; Cisneros-Zevallos, L. The increase in antioxidant capacity after wounding depends on the type of fruit or vegetable tissue. *Food Chem.* **2007**, *101*, 1254–1262.

(45) Kusznierewicz, B.; Smiechowska, A.; Bartoszek, A.; Namiesnik, J. The effect of heating and fermenting on antioxidant properties of white cabbage. *Food Chem.* **2008**, *108*, 853-861.

(46) Flohe, L. The glutathione peroxidase reaction: molecular basis of the antioxidant functions of selenium in mammals. *Curr. Top. Cell. Reg*. **1985**, *27*, 473–478.

(47) Olarte, C.; Sanz, S.; Echávarri, J. F.; Ayala, F. Effect of plastic permeability and exposure to light during storage on the quality of minimally processed broccoli and cauliflower. *LWT-Food Sci. Technol.* **2009**, *42*, 402–411.

(48) Molan, A. L.; Flanagan, J.; Wei, W.; Moughan, P. J. Selenium-containing green tea has higher antioxidant and prebiotic activities than regular green tea. *Food Chem*. **2009**, *114*, 829-835.

(49) Evans, D. A.; Hirsch, J. B.; Dushenkov, S. Phenolics, inflammation and nutrigenomics. *J. Sci. Food Agric*. **2006**, *86*, 2503-2509.

(50) Cho, H.; Seon, M.; Lee, Y.; Kim, J.; Kim, J.; Kim, S.; et al. 3, 3´-Diindolylmethane suppresses the inflammatory response to lipopolysaccharide in murine macrophages. *J. Nutr*. **2008**, *138*, 17-23.

(51) Thimmulappa, R. K.; Mai, K. H.; Srisuma, S.; Kensler, T. W.; Yamamoto, M.; Biswal, S. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res*. **2002**, *62*, 5196–5203.

(52) Institute of Medicine. Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids. Washington, DC: National Academy Press, 2000, pp. 311–315.

(53) Chen, Y-C.; Sosnoski, D. M.; Gandhi, U. H.; Novinger, L. J.; Prabhu, K. S.; Mastro, A. M. Selenium modifies the osteoblast

inflammatory stress response to bone metastatic breast cancer. *Carcinogenesis* **2009**, *30*, 1941-1948.
(54) Rabasco, P.; Aranha, M. M.; Borralho, P. M.; Moreira da Silva, I. B.; Correira, L.; Fernandes, A.; Rodrigues, C. M. P.; Camilo, M. Colorectal cancer: can nutrients modulate NF-κB and apoptosis? *Clin. Nutr.* **2010**, *29*, 42-46.
(55) Christensen, M. J.; Nartey, E. T.; Hada, A. L.; Legg, R. L.; Barzee, B. R. High selenium reduces NF-κB-regulated gene expression in uninduced human prostate cancer cells. *Nutr. Cancer* **2007**, *58*, 197-204.

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FIGURE CAPTIONS

Figure 1. Evolution of pH during cabbage fermentation in absence (NF) or presence of Se (NFSe).

Figure 2. Chromatographic profile obtained by anion-exchange LC-ICP-MS of A) a mixture of Se standards containing 100 μg/L of each Se species; and B) enzymatic extraction of raw cabbage (—), NF cabbage (—) and NFSe cabbage (- - -).

Figure 3. Effect of different concentrations of raw, NF and NFSe cabbage extracts (A); ABG (B); and SeMeSeCys (C) on NO production in LPS-induced macrophages RAW 264.7 cells. Data represent the mean ± standard deviation of a triplicate from three independent experiments. The same letter in the same bar indicates not significant difference (P ≤ 0.05).

Table 1. Effect of Se enrichment on ascorbigen (ABG), indol-3-carbinol (I3C) and indol-3-acetonitrile (I3ACN) content in natural fermented cabbage (*Brassica oleracea* var. *capitata* cv. Megaton)*

Cabbage	ABG (μmol/100g d.m.)	I3C (μmol/100g d.m.)	I3ACN (μmol/100g d.m.)
Raw cabbage	16.43±1.76a	0.10±0.04a	0.24±0.07a
Fermented cabbages			
NF	218.18±4.21c	9.60 ± 0.76b	2.66±0.17b
NFSe	205.26±3.16b	16.70±0.99c	3.00±0.17c

*) Mean value ± standard deviation of three experiments. The same letter in the same column indicates no significant difference ($P \leq 0.05$)

Table 2. Effect of Se enrichment on ascorbic acid, Se biotransformation and water content in natural fermented cabbage (*Brassica oleracea* var. *capitata* cv. Megaton)*

Cabbages	Ascorbic acid (mg/100g d.m.)	Se (µg/g d.m)	SeMeSeCys (µg/g d.m)	Water (%)
Raw cabbage	329.45±8.95c	0.04±0.01a	NDa	91.6
Fermented cabbages				
NF	242.37±8.4b	0.07±0.01b	NDa	91.5
NFSe	229.86±8.50a	1.29±0.04c	0.74±0.02b	91.4

*) Mean value ±standard deviation of three experiments. The same letter in the same column indicates no significant difference (P ≤0.05).

Table 3. Effect of Se enrichment on antioxidant capacity and anti-inflammatory activity of natural fermented cabbage (*Brassica oleracea* var. *capitata* cv. Megaton)*

Cabbage	Antioxidant capacity (μmol Trolox/g d.m.)	Inhibitory potency (IC_{50}) of NO production (μg extract/mL)
Raw cabbage	74.78±0.28a	167.93 ± 16.09c
Fermented cabbages		
NF	131.54±13.95b	83.96 ± 8.50b
NFSe	162.96±3.71c	44.01± 1.39a

*) Mean value ± standard deviation of three experiments. The same letter in the same column indicates no significant difference ($P \leq 0.05$). IC50 is the concentration of cabbage extract (□g /mL) that resulted in 50% inhibition of NO production.

Table 4. Effect of Se enrichment on microbiological quality (cfu/g f.w.) of natural fermented cabbage (*Brassica oleracea* var. *capitata* cv. Megaton)*

Cabbage	Aerobic mesophilic bacteria	Anaerobic mesophilic bacteria	Lactic acid bacteria	Total Coliforms	Faecal Coliforms	Yeasts and moulds
Raw cabbage	5.19±0.11a	4.38± 0.11a	2.42± 0.11a	1.17± 0.18b	<1a	<1 [a]
Fermented cabbages						
NF	6.78± 0.10c	6.75± 0.11c	6.70± 0.14b	<1a	<1a	<1 [a]
NFSe	6.25±0.11b	6.28±0.13b	6.92±0.10c	<1a	<1a	<1 [a]

*) Mean value ± standard deviation of three experiments. The same letter in the same column indicates no significant difference ($P \leq 0.05$)

Figure 1

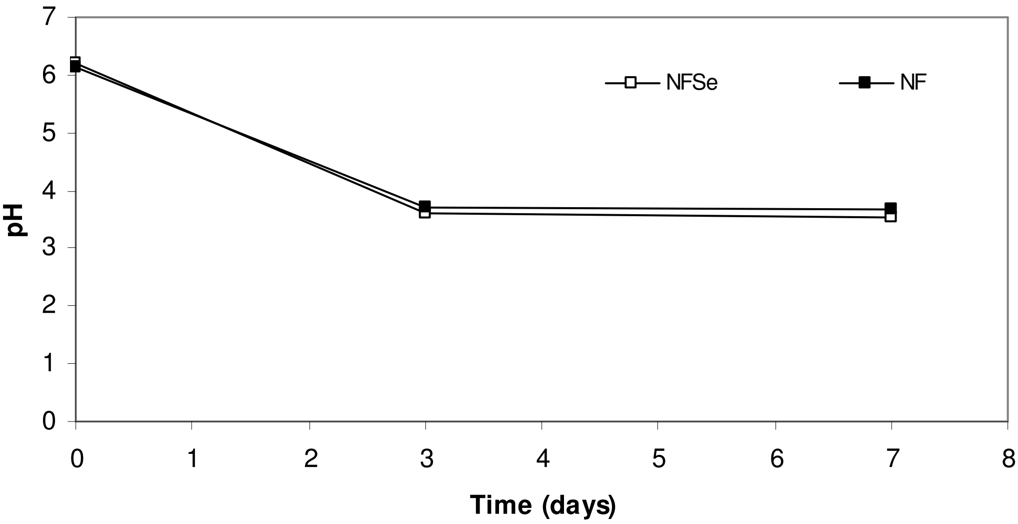
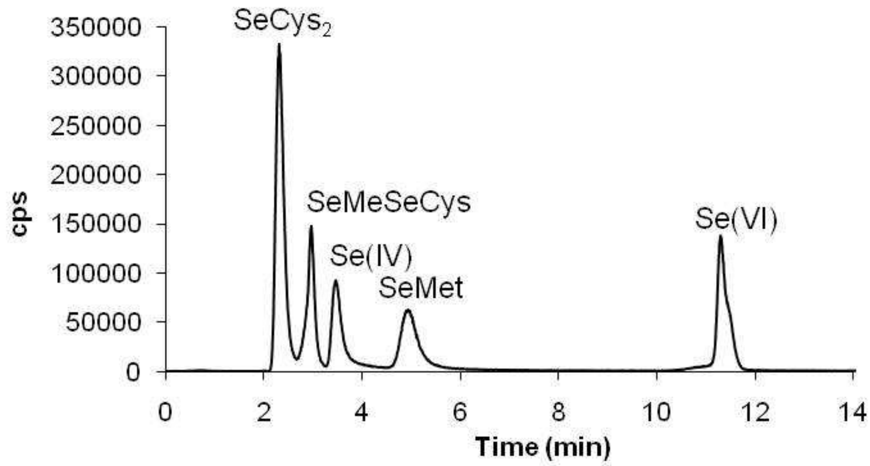


Figure 2

A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SeMeSeCys
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SeMet
Se(VI)
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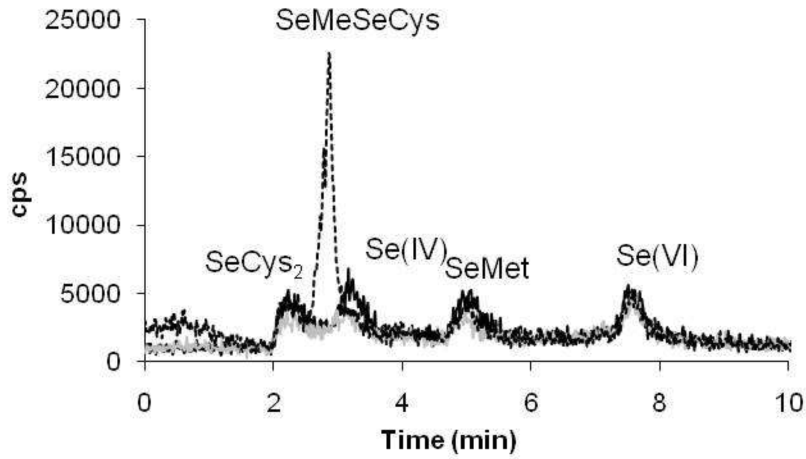
14
Time (min)

B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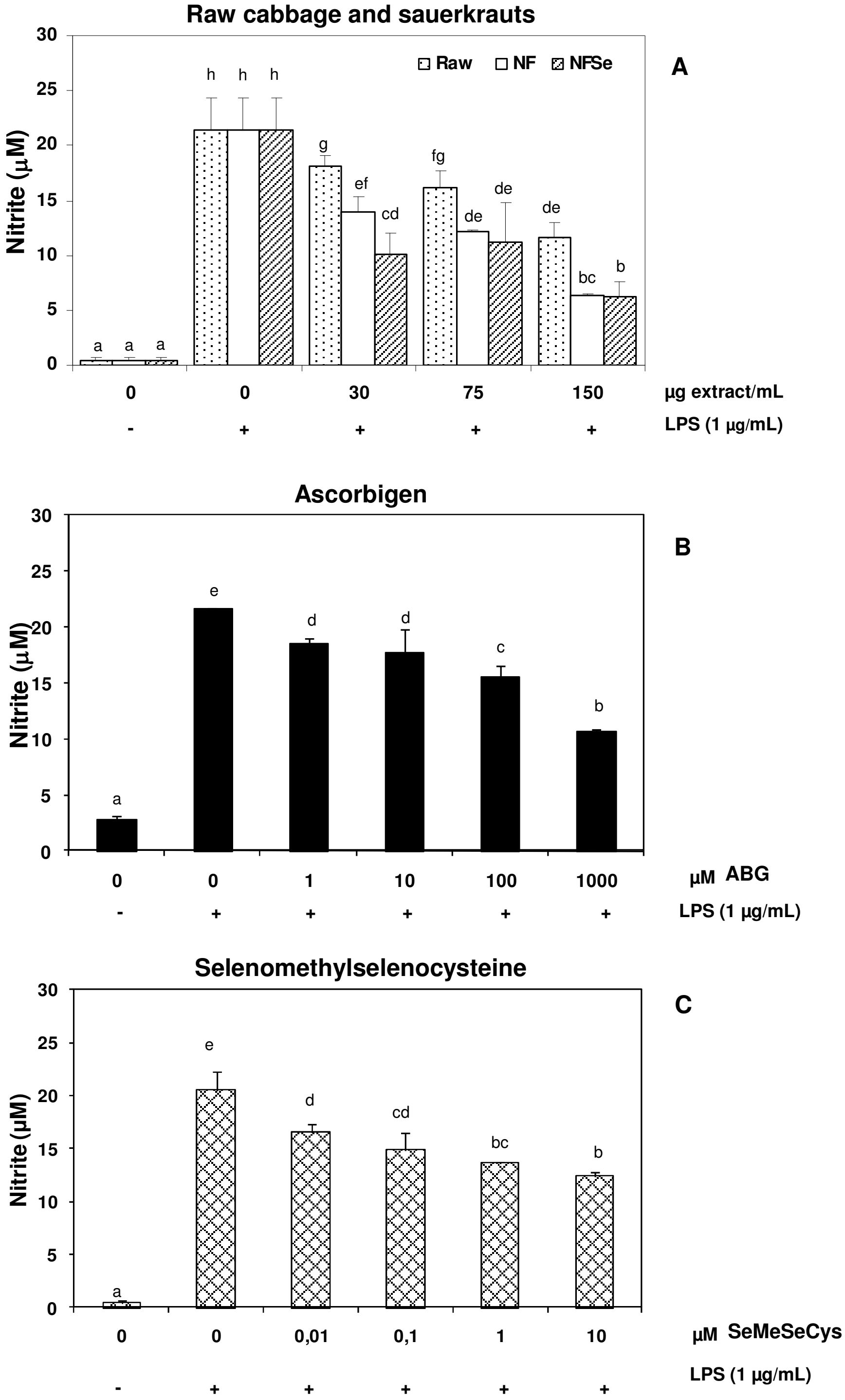
10
Time (min)

Figure 3


Raw cabbage and sauerkrauts
Raw NF NFSe
A
Nitrite (μM)
μg extract/mL
LPS (1 μg/mL)
Ascorbigen
B
Nitrite (μM)
μM ABG
LPS (1 μg/mL)
Selenomethylselenocysteine
C
Nitrite (μM)
μM SeMeSeCys
LPS (1 μg/mL)

TOC

Selenium species in cabbage fermented with selenite solution