Nebraska Soybean Board
FINAL Research Report Form

VB 1/13/2020

Note: Submit this report no later than 90 days after the NSB-funded project officially terminates.
This post-project 90-day time-frame will allow the Lead PI time to complete any final data analysis and a final technical report, plus the drafting of any articles for submission to scientific journals. Note that this completed report will be provided to the National Soybean Checkoff Research Database, (soybeanresearchdata.com).

Project # and Title: #1738
Identification of the Chemical Components of Soybean Seed and Sprout Related to Anti-inflammatory Activity

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Co-PI's & Institutions: Kaustav Majumder (UNL)

Project Date (Including Extension): 10/01/2018 ***to*** 09/30/2019 ***(For example: mm/dd/yyyy to mm/dd/yyyy)***

Total Budget for Project: $ 9,841.48

1. Briefly State the Rational for the Research:

Soybean is one of the great sources of phytochemicals and digestible plant protein sources with health benefits. Health beneficial bioactive peptides are also produced by gastrointestinal digestion of proteins. Some of these display anti-inflammatory activities, which have health-promoting potential to chronic inflammatory disorders associated with gastrointestinal and cardiovascular diseases.
The composition of these anti-inflammatory activities alters during germination process of soybean due to degradation of storage macromolecules and activation of metabolic pathways. Therefore, soybean sprouts likely have altered health beneficial potential than beans. The direct assessment of the anti-inflammatory bioactive compounds from soybean and its sprout will help to improve the therapeutic interventions against chronic metabolic disorders as well as the commercial values of food soybean products. Alternatively, the finding may open a new way of the soybean use as a feedstock for new functional foods or nutraceuticals products.

2. Research Objectives: (copy from project, but keep in a brief bullet format)

The overall goal of the project is to improve health benefits of soybean derived food products on the obesity-associated chronic diseases, which is one of the major threats to the health of the US citizen. To this end, the objective of this proposed project is to identify the molecules in soybean and its sprout which show anti-inflammatory activities in human intestinal cells. We will further identify the genes responsible to the accumulation of these molecules, which will be the targets of genetic-based improvement of soybean seed composition for superior health benefits in the forthcoming projects.
Objective 1: Delineate the changes in chemical composition of soybean during sprouting and gastrointestinal digestion.
Objective 2: Evaluate the anti-inflammatory effect of the germinated whole beans in intestinal epithelial cells after simulated gastrointestinal digestion.
Objective 3: Identification of the chemical component responsible for anti-inflammatory activity and the genes regulating its accumulation

3. General Approach Used and (if applicable) the Nebraska Test Locations:

Objective 1: Soybean seeds were sprouted in the dark for 0, 2 and 4 days representing seed, germinating, and sprout stages, respectively. One set of samples were immediately flash frozen in liquid nitrogen to preserve the metabolites and proteins. Another set were digested by mimicking the gastrointestinal conditions. The chemical composition of the raw and digested samples were measured through multiple analytical techniques including nitrogen determinator (total protein), LC-QTOF (peptide profile), and semi-targeted GC-MS metabolomics analysis.

Objective 2: The anti-inflammatory activity was tested in Caco2 gastrointestinal epithelial cell line to determine the anti-inflammatory activity of the germinated and digested soybean material. The inflammation was induced in the cultured cells by pre-treatment of Lipopolysaccharides (LPS). The expression of pro-inflammatory cytokines and chemokines were measured to quantify inflammation responses.

Objective 3: The correlation between the levels of chemicals and the parameters of inflammatory responses will be tested to identify the chemical components likely to have anti-inflammatory activity via dietary intake.

4. Describe Deliverables & Significance Attained for Each Research Objective:

The research found substantial changes in chemical composition and gastrointestinal protein digestibility of soybean by sprouting. Digested soybean materials show chemical composition diverse from the original material and exhibited anti-inflammatory activities in cultured intestinal epithelial cells. The results of this project revealed a strong health benefit potential of soybean sprouts on the prevention of chronic-inflammation induced metabolic diseases. This finding will impact the consumer behavior to increase the consumption of soybean in their daily diets and hence potentially it will increase the market value. The results obtained through the project will be published in scientific journals and local news to spread the information.

Objective 1: The metabolite analysis in this research revealed the changes of metabolite profile in sprouting soybean seeds. This is useful information to understand the metabolic processes going on in the germinating soybean seeds and have potential to contribute to improve seed quality of soybean. As expected, accumulations of sugars and amino acids were observed following two and four days of sprouting most likely due to the degradation of storage carbohydrates and proteins, respectively. Interestingly, increase in these metabolites did not happen in the soybean sprout materials at Day 2 subjected to the simulated gastrointestinal digestion. This may be related to the susceptibility of these compounds to gastrointestinal digestion which can be affected by the interaction with other chemicals and/or the cellular structures in soybean seeds. Further research is needed to elucidate the mechanism causing the Day 4 specific accumulation of compounds in the digests, which is potentially associated with the bioavailability of chemicals following oral intake.

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4. Describe Deliverables & Significance Attained for Each Research Objective (continued)

Our in-vitro simulated gastrointestinal study showed that the digestibility in the gastric and intestinal phases significantly decreased over the sprouting period. Contrary, the peptide content is significantly increased over the germination period. This is most likely due to the degradation and alteration of soybean proteins during germination. Protein degradation during the germination of soybean seeds produces free amino acids and small peptides, which was shown also by the metabolite profiling results. These results showed that the sprouting of soybean can increase the amount of potentially bioacitive peptides and other compounds.

Objective 2: The most prominent result in the project is that the anti-inflammatory activity was detected specifically in the digested soybean sprouts at the Day 4 in the gastrointestinal epithelial cell line. Significant activity was detected neither in soaked soybeans nor Day 2 sprouts. This result clearly indicated that the sprouting has potential to improve anti-inflammatory activity in soybean.

Objective 3: As the anti-inflammatory activity was observed only in the digested Day 4 sprout, the metabolites and small peptides accumulated specifically at that time point have potential to be bioacitive compounds. Some metabolites including nucleosides, hypoxhantine, and trigonelline are promising candidates of bioactive compounds accumulated in the digests of Day 4 sprouts. We are currently conducting the identification and quantification of peptides in the digests to identify candidates of bioactive peptides which increased their bioavailability specifically at Day 4. The anti-inflammatory activity of these compounds will be tested using authentic materials in the near future to confirm their activity.

5. List where the Project Research Results/Findings were Publicized:

(a) Once the research results are published in a peer-reviewed research journal, they will be distributed via UNL websites and local news to general public including farmers.
(b) The scientific manuscript describing the changes in chemical composition of soybean by sprouting and gastrointestinal digestion and their anti-inflammatory activity on cultured cells are under preparation. This will be published in a scientific journal such as "Journal of Functional foods", "Plos One" etc.

Note: The above boxes will automatically accomodate for your text inputs; HOWEVER, the Final Report comprised of the above listed items must be kept to THREE PAGES. A Technical Report of no more than TEN PAGES (preferably fewer) can be appended to this report.
Submit both reports as a single PDF with this file name format: #XXX > FINAL > Project Title > PI last name

Please email this completed form to the Agriculture Research Division (jmonaghan2@unl.edu) based on the reporting schedule given to you. If you have any questions, please call the ARD at 2-2045 or Victor Bohuslavsky at the Nebraska Soybean Board Office at (402) 432-5720.

Technical Reports

1. Soybean sprouting

Considering the commercial impacts of our experimental results, we decided the experimental condition as close as the dietary practice. The organic soybean grown in the US were obtained from True Leaf Market, Salt Lake City, UT. The seeds were washed twice and soaked in tap water for 16 h at 25°C in the dark. The seeds are then transferred onto a wet paper towel and incubated at 25°C in the dark for up to 5 days. The root became ~7 cm long at the end of incubation period. According to the size for dietary use, we decided to harvest at day 2 and day 4 for further analysis (Figure 1).

Six pools of five sprouts were harvested at day -1 (dry seed), day 0 (right after the 16 h of soaking), day 2 (48 h after soaking), and day 4 (96 h after soaking) and were flash frozen in liquid nitrogen for the analysis of chemical composition. Another 10 g of sprouts were harvested for gastrointestinal digestion without freezing. The experiment was repeated three times.

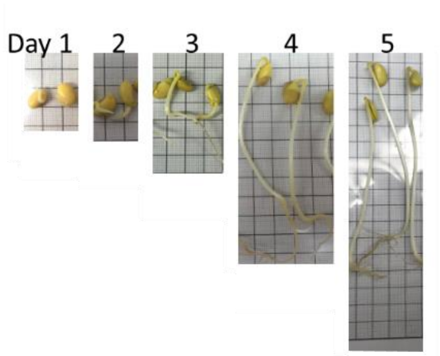

Figure 1: Germination of soybean seeds. Dry soybean seeds were soaked in tap water for 16 h and incubated in dark for 5 days. A picture was taken every 24 h. The sprouts were placed on grid papers with 10 mm square. The seeds at 2 and 4 days of germination were used for following experiments.

2. Chemical profiling of raw soybean materials

The frozen soybean samples were ground to fine powder under liquid nitrogen temperature. Methanol soluble metabolites were extracted from 50 mg aliquots of the materials and analyzed by gas chromatography-mass spectrometry (GC-MS; 7200 GC-QTOF system, Agilent, Santa Clara, CA) using a protocol established recently (Wase, Abshire & Obata, under review). In this analysis, 114 metabolites including six unidentified polysaccharides were detected and the levels of 103 metabolites were significantly affected by soaking and germination in ANOVA analysis ($p<0.05$). The contents of major monosaccharides, namely glucose and fructose, continuously increased to eight times (Figure 2A) while disaccharides and minor sugars (Figure 2B) decreased two to eight times within the 4 days period. Most of the proteogenic amino acids including valine, leucine, isoleucine, proline, serine, threonine, methionine, aspartate, asparagine, glutamine, lysine, and histidine accumulated during sprouting (Figure 2C and D). These metabolic changes are most likely due to the degradation of storage lipids and proteins to produce energy required for germination. The increase of major sugars and free-amino acids can modulate the bio-availability of nutrients and thus enhance the nutritional values of soybean since they are more absorbable during dietary intake than macro storage molecules. In addition to the major changes, some organic acids including malate and fumarate were kept accumulating during the four days period. The levels of pyruvate and 2-oxoglutarate are accumulated following soaking and came back to the same levels as in the dry seeds after two days. These indicate the operation of tricarboxylic acid cycle in two distinct flux modes during the germination process.

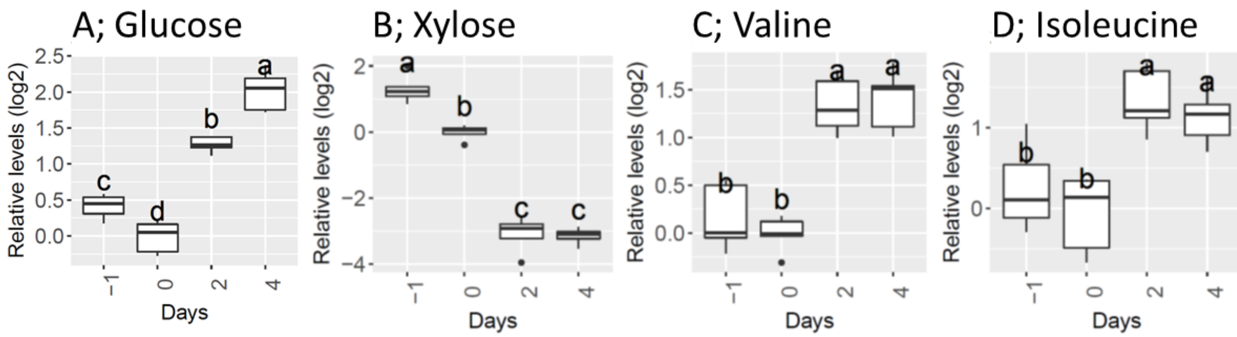

Figure 2: Levels of metabolites in soybean and its sprout during the germination. Relative levels of metabolites were determined by GC-MS based metabolite profiling in dry beans (Day -1), beans soaked for 16 h (Day 0), and sprouts at 2 and 4 days of germination. Letters indicate the results of Tukey HSD test (p<0.05). A; glucose, B; xylose, C; valine, D; isoleucine.

3. Gastrointestinal digestion of the soybean material

The gastrointestinal digestibility of the sprouted soybean samples was carried out *in-vitro* by simulated gastrointestinal study. The sprouted soybeans are first digested with alpha-amylase as a part of the oral digestion, followed by pepsin at pH 3 to mimic the stomach digestion, and pancreatin and bile salt at pH6.5 to mimic the intestinal digestion.

- Digestibility of soybean derived proteins reduced during sprouting.

Changes in digestibility or gastrointestinal digestibility (amount of peptide bond breakage due to gastrointestinal enzymatic hydrolysis against total amount of peptide bond in proteins) affect the amounts of free amino acids and peptides in the small intestine. Our in-vitro simulated gastrointestinal study showed that the digestibility in the gastric and intestinal phases significantly decreased over the sprouting period (0 to the Day4, Figure 3A & B). This is most likely due to the degradation and alteration of soybean proteins during germination. Protein degradation during the germination of soybean seeds produces free amino acids and small peptides, which has been partly shown by the metabolite profiling results reported in the Figure 2. The selected degradation could cause the changes in protein composition in soybean material and affected gastrointestinal digestibility. Germination also likely alters the structure of the soybean storage proteins, mostly affecting the cleave sites of the pepsin (gastric proteolytic enzyme) and the pancreatin (intestinal proteolytic enzyme mixture). Additionally, these results indicate the possibility of the production of novel and unique peptide sequences after 4 days of germination, which might not be produced in the gastrointestinal tract without germination.

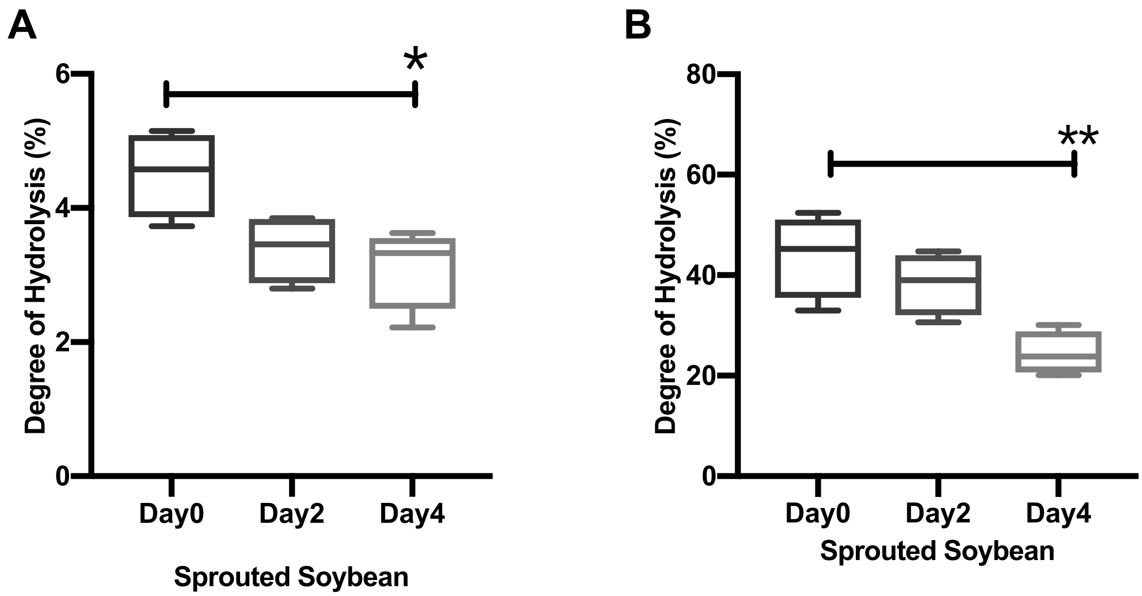

Figure 3: The degree of hydrolysis (DH: %) of the sprouted beans at 0, 2 and 4th day. (A) the DH in the gastric phase, significantly decreased after 4 days of sprouting. (B) the DH in the intestinal phase also showed a decreasing trend in the digestibility significantly decreased after 4 days of sprouting. * indicates *P< 0.05 and* ** indicates *P< 0.01*, results were obtained from n=4 independent experiments.

4. Chemical profiling of digested soybean materials

- Changes of metabolite contents in the digested soybean materials by sprouting

The contents of metabolites in the gastrointestinal digests of soybean sprout materials were analyzed by a GC-MS based metabolomics approach. In this analysis, 120 metabolites were detected and the levels of 46 metabolites were significantly changed depending on the day of sprouting through ANOVA analysis (p<0.05). Majority of proteogenic amino acids are accumulated specifically in the Day4 sprouted and digested samples (for example, valine and isoleucine; Figure 4A and B, respectively). This is different from the results in the raw materials in which amino acids started accumulating from Day2 and stayed at the same levels in Day4 (Figure 2). Similar trend was observed in the levels of major sugars including fructose and glucose (Figure 4C). These different tendency in the levels of amino acids and sugars between digests and raw materials might be due to the changes in the susceptibility of these compounds to gastrointestinal digestion which can be affected by the interaction with other chemicals and/or the cellular structures in soybean seeds. Further research is needed to elucidate the mechanism causing the Day4 specific accumulation of compounds in the digests, which is potentially associated with the bioavailability of chemicals following oral intake. Day4 specific accumulations were also observed in nucleosides (inosine, uridine, and guanosine; Figure 4D), hypoxanthine, citrulline (Figre 4E), trigonelline (Figure 4F), and some minor sugars including lyxose, xylitol, and altorose. Disaccharides and minor sugars in the digests showed decreasing tendency within the four days period following the contents in the raw materials (Figure 4G). Organic acids show minor changes throughout sprouting except for malate (Figure 4H) and glycolate which accumulated specifically at the Day4.

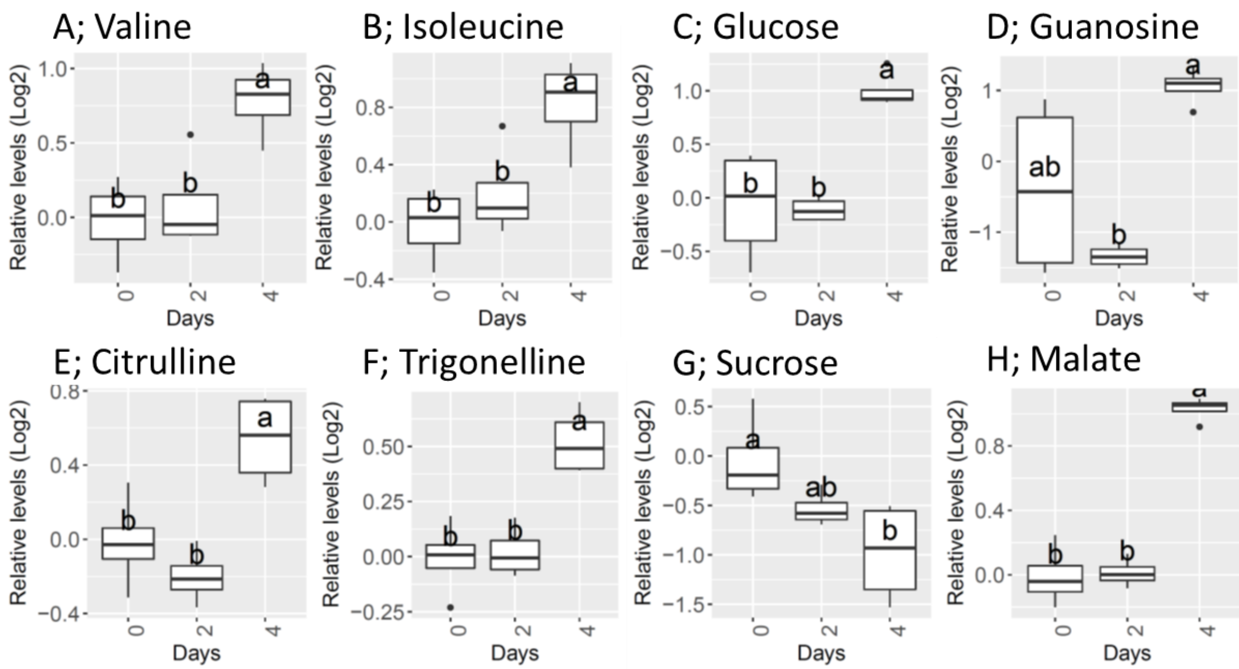

Figure 4: Levels of metabolites in the gastrointestinal digests of soybean and its sprout harvested during the sprouting period. Relative levels of metabolites were determined in the gastrointestinal digests of beans soaked for 16 h (Day 0), and the sprouts at 2 and 4 days of germination by the GC-MS based metabolite profiling. Letters indicate the results of Tukey HSD test (p<0.05). A; Valine, B; Isoleucine, C; Guanosine, D; Guanosine, E; Citrulline, F; Trigonelline, G; Sucrose, H; Malate.

- Peptide contents in the digested soybean materials

The degree of hydrolysis declined after germination and the digestibility significantly decreased after Day4 germination in both gastric and Intestinal Phase (Figure 3). However, with these results, we still did not know that the actual amount of peptide in the hydrolysate. It is to remember that the degree of hydrolysis measurement process measures the peptide production during the digestion process does not quantify the amount of peptides in the sample. Thus, we need to determine the peptide content in these samples to evaluate the combined effect of germination and gastrointestinal digestion. The peptide content in the sprouted and digested soybean samples was measured by a fluorometric based peptide assay method using a Pierce™ Quantitative Fluorometric Peptide Assay kit. Quantitation of peptides via this method was achieved using an amine-reactive fluorescent detection reagent that specifically labels the N-terminus of peptides. The fluorescently labeled peptides were then detected at Ex 390nm/Em 475nm. This assay is very sensitive and only requires 10 µL of the sample. The results from this assay indicated that the peptide content is significantly increased in the Day4 sprouted and digested samples (Figure 5), indicating that the combined proteolytic action of the germination process and gastrointestinal digestion process. Thus, it is evident from this data that the germination process initiates the proteolytic process and produces shorter peptides, however, due to the same reason the gastrointestinal enzymes have fewer specific sites and decreasing the digestibility. It is an exciting finding and perhaps for the first time exhibit the synergistic effect and the relationship between germination and gastrointestinal digestion to produce peptides from plant-derived food proteins. Additionally, it should be also noted that the structure and sequence of the peptides produced after gastrointestinal digestion in the sprouted soybeans could be very different from un-sprouted soybeans, this difference in peptide structure and sequence could modulate the health beneficial biological activities.

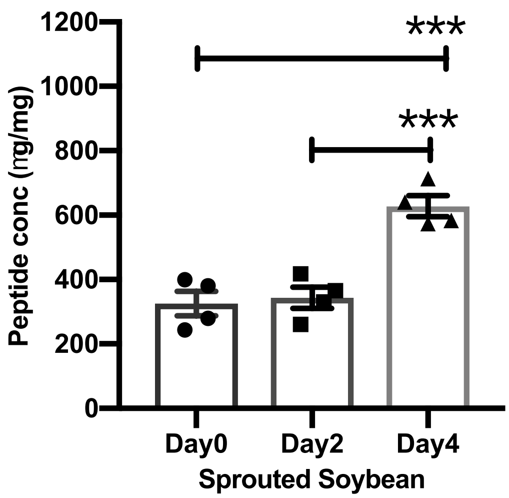

Figure 5: The peptide content of the sprouted beans (0, Day2 and Day4) hydrolysate after simulated gastrointestinal digestion. The peptide content significantly increased in the Day4 sprouted-digested samples compare to Day0 and Day2. *P*<0.01. *** indicates *P< 0.001*, results were obtained from n=4 independent experiments.

5. Anti-inflammatory effects of sprouted soybeans after gastrointestinal digestion.

The soluble fraction was separated by centrifugation after gastrointestinal digestion, to evaluate the anti-inflammatory activity of the sprouted soybean hydrolysate (SSH). The anti-inflammatory activity was then tested on gastrointestinal epithelial cells (Caco2 cells). Briefly, Caco2 cells were seeded in a 48 well plate (50,000 cells/well) with EMEME media containing 20% fetal bovine serum (FBS) and antibiotics. After 6-7 days, the cells become 80% confluent, and we used these cells for further analysis. The media was changed every 48 hrs during this growth period. After the cells reach 80% confluency, cells were quickly washed with Hanks' Balanced Salt Solution (HBSS), and low serum (1% FBS) media was used to conduct the further experiment. The Caco-2 cells were then pre-treated with sprouted soybean hydrolysate (SSH: 1000mg/mL) for 2 hrs, and then inflammation was induced in these cells by Interleukin-1β (IL-1β) at a concentration of 25ng/mL and incubated for next 24 hrs. After the incubation period, the culture media was collected to evaluate the expression of pro-inflammatory cytokine IL-8 through ELISA, using a human Sandwich ELISA kit specific to IL-8 (Thermofisher, USA). The results indicates that Day4-sprouted samples after gastrointestinal digestion can exhibit anti-inflammatory effect by significantly reducing the expression of pro-inflammatory cytokine IL-8 (Figure 6).

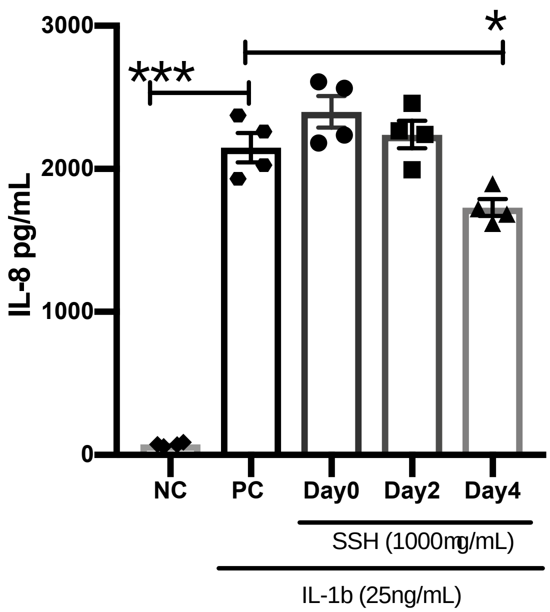

Figure 6: The effect of sprouted soybean hydrolysate (SSH) on IL-8 production in intestinal Caco2 cells. The IL-8 expression was significantly increased in the positive control (PC) group after IL-1β treatment compare to the negative control (NC) group. However, the increased expression of IL-8 was significantly decreased only after Day4-SSH treatment, indicating the anti-inflammatory activity of the Day4-SSH sample. * indicates *P< 0. 05 and* *** indicates *P< 0.001*, results were obtained from n=4 independent experiments.

7. Identification of the candidate bioactive compounds with anti-inflammatory activity

Since anti-inflammatory activity was observed only in the Day4 SSH sample, the compounds accumulated specifically found in the Day4 SSH sample likely exhibit the observed bioactivity. Although most of amino acids and major monosaccharides accumulated at Day 4, it is highly unlikely these amino acids have anti-inflammatory activities considering general availability of them. It should be noted that some nucleosides including uridine, adenosine, and inosine, and hypoxanthine, which composes a part of inosine, also accumulated specifically at Day 4 in the digested sprouts. Another interesting candidate is trigonelline, which also accumulated in the Day-4 digests. Trigonelline is reported to be accumulated in a crop plant, *Moringa oleifera* (commonly called as drumstick; Mathur and Kamal, 2018), which is reported to have anti-inflammatory health benefits (Sulaiman et al, 2009). Additionally, the unique peptides that may produce in the Day4-sprouted samples after gastrointestinal digestion could contributes towards the anti-inflammatory activity.

Conclusions

In this project, we could successfully show the substantial alteration in the chemical composition in germinating soybeans and their gastrointestinal digests. The digest of sprouts at Day 4 specifically showed anti-inflammatory activity in the cultured gastrointestinal epithelial cells. These results indicated the potential of soybean sprouts as a functional food to prevent chronic inflammatory disorder often associated with obesity. As the anti-inflammatory activity was observed only in the Day 4 sprout, the compounds specifically accumulated in the digest at this time point likely have anti-inflammatory bioactivity. These compounds include nucleosides, trigonelline, and related compounds, some of which have been suggested to have anti-inflammatory activities. Additionally, identification and quantification of individual short peptides in the gastrointestinal digests of soybean materials has been conducted although the data analysis is still underway. The peptides with increased bioavailability in the digests of Day 4 sprout are also promising bioactive compounds. Further experiments using authentic compounds will elucidate the anti-inflammatory activities of these compounds. Additionally, the experiments using animal models are being conducted in the FY20 period to investigate the in vivo anti-inflammatory activities of soybean sprouts. Scientific and general reports are under preparation to disseminate the results showing health beneficial potential of soybean sprouts.

References

Mathur M, Kamal R (2012) Studies on trigonelline from Moringa oleifera and its in vitro regulation by feeding precursor in cell cultures. Brazilian Journal of Pharmacognosy 22(5): 994-1001

Sulaiman MR, Zakaria ZA, Bujarimin AS, Somchit MN, Israf DA, Moin S (2008) Evaluation of Moringaoleifera Aqueous Extract for Antinociceptive and Anti-Inflammatory Activities in Animal Models, Pharmaceutical Biology, 46:12, 838-845