An Roinn Talmhaíochta,
Bia agus Mara
Department of Agriculture,
Food and the Marine

13F510 - Fungal biofactories: Improved delivery of natural selenium from the cultivated mushroom (*Agaricus bisporus*)

Final Report

This project was funded under the Department of Agriculture, Food and the Marine Competitive Funding Programme.

SUMMARY

The aim of this proposal is the characterisation of Se speciation in mushrooms and their by-products and the optimisation of oral bioavailability of Se from mushroom products using nanoparticle drug delivery technology to support health claims for supplementation.

Results and impact

Using HPLC-PDA concentrations of seleno amino acids in mushrooms were tested in industrial mushrooms and showed a concentration range between predominant species identified was selenocystine at 616±23 µg/g dry. Modest amounts of SelenoMethionine were found, at 18±9 µg/g dry. This finding points to the potential use by the food industry to use the results in already approved health claims, develop Se enriched supplements for human or veterinary purposes and use inexpensive analytical techniques to monitor selenoaminoacid content in crop.

An encapsulated Selenium drug delivery technology has been developed in this project. Initially the formulation used chemically pure compounds. These experiments confirmed the absence of toxicity in the concentration range, stability for storage purposes of the formulation. Transport studies in-vitro and ex-vivo confirmed the ability to produce a slow release and to resist liver enzymes. In-vivo experiments indicated the possibility that the Selenoaminoacids are being transformed through epithelial passage. These novel results can be used by researchers and industry to produce particles (over the < 100nm) to improve delivery of bioactives.

Further to that the same formulation was performed using mushroom origin chitosan with results demonstrating the feasibility of using food grade ingredients. This result will enable research and development in academia and industry to use similar formulations using mushroom origin chitosan.

Finally, a transport enhancer, Trymethylchitosan, based on fungal chitosan has been developed, opening the possibility of using it in pharmaceutical research to improve oral delivery of Se-amino acids or other peptides. This result will enable research in the area of fungal chitosan derivatives of use to the Irish mushroom industry.

KEYWORDS

Selenium; mushrooms; formulation.

ACRONYM	SELNUTR
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Section 1 - Research Approach & Results

Start Date

01 March 2014

End Date

31 October 2018

Research Programme

Food Institutional Research Measure

TRL Scale

TRL 4: Technology validated in lab

NRPE Priority area

Food for Health

Total DAFM Award

€474,627.50

Total Project Expenditure

€365,362.39

Rationale for undertaking the Research

While a large number of molecules from mushrooms with possible health promoting properties have been studied, the health claims ascribed to mushrooms and recognised by the EU arising from Se content are the most solid evidence for the development of a health claim for mushrooms. Se levels in soil vary in different geographical areas, Ireland being one of the countries in Europe with high environmental concentrations. Appropriate agricultural practices can further increase Se concentration in mushrooms (Coelho, 2012 & Thiry et al, 2012). Se supplementation is problematic due to the narrow range between therapeutic and toxic concentrations. Encapsulating into a nanoparticle using suitable excipients may overcome this by creating an oral controlled release formulation.

Methodology

The analysis of Seleno amino acids in the mushrooms used enzymatic extractions followed by reverse phase chromatography. This was complemented with AA-GFA, ICP-OES and benchmarked against external ICP-MS results. Samples with physiological levels of Seleno amino acids in the mushrooms were analysed using ICP-MS and LC-qToF. The toxicity of Seleno amino acids was assessed using the MTS and Lactate dehydrogenase assays in Caco-2 and HepG2 cells. The antioxidant activity of Selenium species was evaluated using the ROS and the GSH assays. All procedures involving animals were carried out under the guidelines outlined by the (NC3R) and adhered to EU Directive 86/609/EEC for use of animals in experiment and other scientific purposes. Transport properties of Seleno amino acids was assessed employing Using chambers across rat colonic and intestinal mucosae and intestinal tissue. The toxicity was assessed in those tissues using Transepithelial electrical resistance (TEER), LDH assays and histology. The potential of SeMet in oral rehydration therapy for acute diarrhoea used electrophysiology experiments of rat colonic mucosae exposed to Seleno amino acids and diarrheic agents. The

delivery formulation was developed using ionotropic gelation. The physicochemical characterisation of particles employed DLS and confirmed using SEM. FTIR spectroscopy was used to confirm binding of the particles. Cytotoxicity using MTS standard assay was employed. Controlled release studies used the bag dialysis diffusion technique. In-vivo and ex-vivo instillation experiments were employed to test the mushroom-based formulation. Transport was assessed using LC-qToF and TEER. Chitin/chitosan production employed microwave assisted extraction and deacetylation. Mushroom chitin and chitosan were characterised using NMR (1H and 13C), Gel permeation chromatography and FTIR. Histology assessed non-toxicity of the mushroom chitosan formulation and ex-vivo studies confirmed stability against GIT and liver enzymes.

The transport enhancement ability of chitosan derivatives was assessed employing Using chambers and TEER. Design of Experiments was employed to reduce experimental needs and optimise.

Project Results

- An HPLC-UV/Vis as an alternative analytical technique for the analysis of Se-amino acids in mushroom extracts has been developed.
- The major components of Selenium speciation have been identified as Selenomethionine (SeMet) and Selenocysteine (SeCys2). Literature suggests as well the presence of smaller concentrations of methyl Selenomethionine, however those haven't been identified.
- Sampling of mushroom extract from baseline production and from fortification experiments has been conducted.
- A methodology to produce chitosan from A. bisporus has been developed.
- A 1H-NMR method was developed and optimized to determine the degree of deacetylation (DDA%) of commercial and extracted chitosan.
- The study indicated that fungal chitosans could be produced with equivalent DDA% to that of CL113 (a standard pharmacy industry specification chitosan) (86±6 vs 85±4 %).
- A further modification of chitosan investigated, whereby the free amine groups present on the polymer backbone were subjected to exhaustive methylation to give rise to TMC. TMC has been assessed in vitro and showed potential application as a permeation enhancer.
- Ionotropic gelation experiments developed a particle formulation with target size and stability for oral delivery, low particle size distribution, and an encapsulation efficiency above the targets for scaling up for industrial application. The formulation used CL113 chitosan and zein to encapsulate Se-amino acids.
- Controlled release experiments using a sequential diffusion cell experiment in Simulated Gastric Fluid (SGF) followed by Simulated Intestinal Fluid (SIF) indicated that the main processes controlling release of the Se-amino acids in the nanoparticle are those related to abrasion and swelling of the material.
- There is a significant amount of Se-amino acids released in the SGF step, indicating that the formulation will need to have enteric protection so to avoid the destruction of the nanoparticle and the oxidation of the Se-amino acids.
- Both MSC and SeCys2 nanoparticles were tested for accelerated temperature storage showing a large stability at high temperatures.
- The Se-amino acid (SeMet, MSC or SeCys2) used to load the NPs did not have an effect on the formulation stability. * SeMet, SeCys and MeSeCys remain intact following incubation in excised rat liver & intestinal homogenates and intestinal fluid. This is indicated through the measurement of the three species at varying time points using RP-HPLC, with no significant reduction in Se quantity after a 2-hour incubation period.
- No significant toxicity was found in the range 10-200 μM.
- A transcellular mechanism of transport is most likely to dominate the transport of Se-amino acids. The apparent permeability coefficient of SeMet and SeCys was shown to be circa10-4cm/s across isolated rat intestinal tissue mucosae.

- Ex-vivo transport showed that jejunum dominates the transport of Se-amino acids.
- The comparison on the ex-vivo uptake of Se-amino acids and their respective nanoparticle formulation showed a slower release of the nanoparticle against the non-formulated Se-amino acid.
- Se-amino acid loaded nanoparticles oral transport in vivo was evaluated against their native Se-amino acid with nano formulations showing a consistently controlled release.
- Administered doses below the toxicity levels indicated that the nanoparticle formulation may contribute to a reduction in this toxicity.

Section 2 - Research Outputs

Summary of Project Findings

Industry

Industry stakeholders have benefited from the project with the development of a rationale for formulation of Selenium supplements from a pharmacological and food technology perspective. Industry has also benefit from the results and methodologies outlined in this project in regard to the extraction chitin and the production of chitosan from mushroom material. Finally, the development of chitosan based transport enhancers benefits the pharmaceutical industry that obtains a reliable, relatively homogeneous source of chitosan and chitosan derivatives.

Consumers

This project has brought the potential for Se supplementation up to the stage before testing with humans. Reduced toxicity, stability and controlled release seem to give a good promise for a natural mushroom-based Selenium supplement.

Summary of Staff Outputs

Research Output	Male	Female	Total Number
PhD Students	0	1	1
MSc Students	1	1	2

Summary of Academic Outputs

Research	No.	Details
Publications in Peer Reviewed Scientific Journals	2	2 peer reviewed articles have been published in the area of formulation of seleno amino acid supplements. 2 more articles are remaining to discuss the final mushroom formulation and the ex-vivo results.
Peer Reviewed Conference Papers	15	The project has been extensively presented in conferences and workshops both nationally and abroad.
PhD Theses	1	The PhD thesis "Formulation and in Vitro Characterisation of Fungal Chitosan Nanoparticles Coated With Zein for Improved Oral Delivery of Seleno amino Acids" was successfully completed by Giuliana Vozza.
Masters Theses	2	1. The MSc thesis "Improved delivery of natural selenium from the cultivated mushroom (Agaricus bisporus)" was successfully completed by Shane Forde 2. The MSc thesis "Evaluation of the in vitro cytotoxicity and ex vivo intestinal permeability of selenium-containing amino acid species" was successfully completed by Ciara McDonald

Intellectual Property

The UCD and the TU Dublin partners have been in continuous contact with their respective innovation offices to identify potential IP to protect. At the end of the project the coordinator engaged with Hothouse in an exercise to review the possible elements of novelty.

As a result of that a plan to develop further IP in the development of transport enhancers from chitosan derivatives was agreed by the consortium. This has led to 2 proposals in this area. Conversation with industrial partners indicate the interest in developing further knowledge in the extraction of chitosan and Se-amino acids for food use with little appetite for pharmaceutical application due to regulatory issues. The consortium intends to pursue the research on Pharmaceutical use chitosan derivatives by searching a partnership with a pharmaceutical company. The development of Se-amino acid supplement formulation and mushroom enrichment will be pursued in research proposals with industrial partnerships, searching for co-funding.

Summary of other Project Outputs

Project	Details	Total No.
New Products	A Selenium supplement based on Seleno amino acids encapsulated in chitosan both from mushroom origin with technological equivalence to pharmaceutical industry standards.	1
New Industry Collaborations Developed	A collaboration with industrial partners to develop chitosan from mushroom origin.	1

Potential Impact related to Policy, Practice and Other Impacts

Impact	Details
Industry	This project may open a potential impact to valorise mushroom production waste looking at the recovery of Selenium. This provides new avenues to improve the already good sustainability of this industry.

Dissemination Activities

Activity	Details
Seminars at which results were presented	Periodical Seminars (annually) where the results were presented to industrial stakeholders were held, engaging Anabio, Monaghan Mushrooms and AllTech. At the end of the project two seminar presentations were made by the project team to the industrial stakeholders (June and August 2018). This promoted a frank discussion on the potential future research collaboration opportunities. The results of the project were presented at the following national scientific meetings: • (9-10th September 2015). Poster presentation. Conway Festival (UCD). • (14th December 2015). Oral presentation. Annual Food Research Conference (Teagasc, Moorepark). The results of the project were presented at the following international scientific meetings: • (14-17th July 2015). Poster presentations, Delivery of Functionality in Complex Food Systems (DOF),Paris. • (10-12th Nov 2015). Oral presentation. The European Federation of Food Science & Technology (EFFoST,) Athens. • (July 17-20th 2016). Poster presentation. Controlled Release Society 43rd Annual Meeting, Seattle, Washington • (April 2016). Poster presentation – United Kingdom and Ireland controlled release society (UKICRS) symposium. Cork. • (August 2016). Poster presentation IUFoST 18th World Congress of Food Science and Technology, Dublin. • (Dec 2017), Oral presentation. Innovations in Encapsulation, Royal Society of Chemistry (RSC) conference, London, UK. • (Nov 2017) Poster presentation, American Association of Pharmaceutical Scientists (AAPS) conference, San Diego, California. • (Nov 2017), Poster presentations, The European Federation of Food Science & Technology (EFFoST) conference, Sitges, Spain. • (June 2018), United Kingdom and Ireland controlled release society (UKICRS) Workshop & Symposium, Belfast, NI.

Knowledge Transfer Activities

Identify knowledge outputs generated during this project	The key learning of the project is the applicability of prior knowledge in pharmaceutical drug delivery research on the development of food origin bioactive formulations, demonstrated in this project to the in-vivo level of a formulation developed with food origin ingredients. An Irish knowledge-how has been developed within the collaborating team (UCD, TUDublin) in the optimisation of peptide delivery formulations. The prior knowledge on the ability of chitosan and zein to encapsulate peptides has been demonstrated further with Seleno amino acids. A simple HPLC-PAD method has demonstrated to be satisfactory to analyse Seleno amino acid species in mushrooms cultivated employing Se enriching techniques. Mushroom origin chitosan has been produced to mimic the properties of pharmaceutical grade chitosan (CL113) successfully. A new methodology to develop a transport enhancer employed in pharmaceutical peptide delivery from mushroom sources has been proposed. There is evidence that Seleno amino acids might be transformed upon passage by the intestinal epithelial. The potential use of Seleno amino acids for oral rehydration of patients with acute diarrhoea has been assessed.
Identify any knowledge transfer activities executed within the project.	The project proposal preparation stage was benefited from collaboration and discussion with industry partners that indicated the relevance of the topic and the potential areas of industrial interest in the project. A stakeholder committee was installed at the initiation of the project including relevant industry partners. The committee met on an annual basis and discussed the project progress as well as the future direction of the project. Of specific interest for the research project where the guidance in terms of the Selenium metabolism and analysis provided by AllTech, the access to mushroom materials from enriching experiments from Monaghan Mushrooms and insight on bioaccumulation as well as the identification of milestone formulation properties (encapsulation efficiency, uniformity of properties) that directed the research effort. At the end of the project two seminar presentations were made, the first one to management of AllTech (x2 present) and the second one to the research hub of Monaghan Mushrooms (c 50 researchers in the presentation). Following the end of the project one further meeting took place in in Dunboyne focused on Selenium research and a number of meetings with Monaghan Biosciences took place focused on the area of chitin, chitosan and derivatives.
List any impacts resulting from the knowledge transferred during the project.	There is anecdotal evidence of the impact resulting from the knowledge transfer of the project: 1. Within the timeline of the project Monaghan Mushrooms has developed a Selenium enriched mushroom with a health claim to improve immune system. 2. During the participation in the project, an IRC Enterprise Fellowship from Monaghan Mushrooms and supervised by TU Dublin has develop further studies on the chitin/chitosan extraction from mushroom by-products. 3. Following the end of the project, Monaghan Biosciences has engaged with TU Dublin in the preparation of research proposals for the development of mushroom derived chitin/chitosan and other derivatives.

Section 3 - Leveraging, Future Strategies & Reference

Leveraging Metrics

None

Future Strategies

During this project Dr Jenna Warby (Scientific Officer, Monaghan Biosciences) has underwent a PhD thesis funded by IRC and supervised in TU Dublin in the area of valorisation of mushroom by-products. It is expected that this collaboration will continue.

Presently two proposals are in preparation in the area of chitosan production and chitosan derivatives production from mushroom by-products for an MSCA-CareefFit application and for the SFI New Frontiers programme respectively.

The area of Selenium extraction and further development of formulations through green technologies has the potential to develop a direct impact to the industry, developing a sustainable Selenium source for the human supplement or veterinary application. A partnership with veterinary research is another strategy to further develop the area of Se supplementation from mushroom by-products.

Project Publications

1. Mc Kinley C.B.A., Brayden, D. J and Ryan, S. M. (9-10th September 2015). Poster presentation: “Estimation of therapeutic index for various Selenium species in the development of an oral drug formulation”. Conway Festival (UCD).
2. Mc Kinley C.B.A., Brayden, D. J and Ryan, S. M. (14th December 2015). Oral presentation. “In vitro intestinal permeability and cytotoxicity of selenium-containing amino acids species”. Annual Food Research Conference (Teagasc, Moorpark).
3. Giuliana Vozza, Minna Khalid, Hugh J. Byrne, Sinead Ryan and Jesus Frias (14-17th July 2015). Poster presentation - “Preparation and characterisation of selenomethionine-loaded chitosan nanoparticles for oral delivery” Delivery of Functionality in Complex Food Systems (DOF,Paris).
4. Giuliana Vozza, Minna Khalid, Hugh J. Byrne, Sinead Ryan and Jesus Frias (10-12th Nov 2015). Oral presentation –“Encapsulation efficiency and physico-chemical properties of a seleno methionine-loaded chitosan nanoparticles formulation for oral delivery” The European Federation of Food Science & Technology (EFFoST, Athens).
5. Vozza, G., Khalid, M., J. Byrne, H., Ryan, S. and Frías, J. (2016). Nutrition - Nutrient delivery. In: Nanotechnology in the Food Industry, 5th ed. Oxford, United Kingdom: Elsevier, ISBN: 978-0-12-804304.
6. Mc Kinley C.B.A., Brayden, D. J and Ryan, S. M. (July 17-20th 2016). Poster presentation. In vitro intestinal permeability and cytotoxicity of selenium-containing amino acids species. Controlled Release Society 43rd Annual Meeting, Seattle, Washington 2016; Abstract 1865.
7. Mc Kinley C.B.A., Brayden, D. J and Ryan, S. M. (August 21-25th 2016). Poster presentation-IUFOST (Dublin, 2016): To investigate the antioxidant potential of DL-Selenomethionine in vitro and effects on lactate dehydrogenase release ex vivo for oral formulation development. IUFoST 18th World Congress of Food Science and Technology; Abstract 1073.
8. Vozza G., Khalid, M., Byrne, HJ, Ryan, SM and Frias, JM (April 2016). Preparation, optimization and characterization of selenium- loaded chitosan nanoparticles for oral delivery Poster presentation – United Kingdom and Ireland controlled release society (UKICRS) symposium.

9. Vozza G., Khalid, M., Byrne, HJ, Ryan, SM and Frias, JM (August 2016). Preparation and characterization of selenium loaded chitosan nanoparticles for oral delivery. Poster presentation IUFoST 18th World Congress of Food Science and Technology.
10. Giuliana Vozza, Minna Khalid, Hugh J. Byrne, Sinead Ryan and Jesus Frias. Oral presentation – "Methylselenocysteine loaded chitosan:zein nanoparticles: Formulation, characterisation, and in vitro evaluations" Innovations in Encapsulation, Royal Society of Chemistry (RSC) conference, Dec 2017, London, UK.
11. Giuliana Vozza, Minna Khalid, Hugh J. Byrne, Sinead Ryan and Jesus Frias. "Formulation, characterisation and in vitro assessment of Selenocystine loaded chitosan nanoparticles for oral delivery" American Association of Pharmaceutical Scientists (AAPS) conference, Nov 2017, San Diego, California. Poster presentation.
12. Giuliana Vozza, Hugh J. Byrne, Sinéad Ryan and Jesús Frías Poster presentation – "Isolation and characterisation of chitin and chitosan from the cultivated mushroom Agaricus bisporus" The European Federation of Food Science & Technology (EFFoST) conference, Nov 2017, Sitges, Spain.
13. Giuliana Vozza, Minna Khalid, S. Forde, Hugh J. Byrne, Jesus M. Frias, Sinéad M. Ryan, "Fungal chitosan nanoparticles coated with zein for improved oral delivery of selenocystine" The European Federation of Food Science & Technology (EFFoST) conference, Nov 2017, Sitges, Spain.
14. Vozza, G., Khalid, M., Byrne, H.J., Ryan, S. and Frias, J., 2017. Nutrition—nutrient delivery. In Nutrient Delivery (pp. 142). Academic Press.
15. Shane Forde, Sinead Ryan, David J. Brayden.IN VITRO AND EX-VIVO TESTING OF SELENIUM INTERACTIONS WITH INTESTINAL EPITHELIA: TOWARDS AN ORAL DELIVERY FORMULATION. 2018 UKICRS Workshop & Symposium, Belfast, NI June 2018.
16. Vozza, G., Danish, M., Byrne, H.J., Frías, J.M. and Ryan, S.M., 2018. Application of Box-Behnken experimental design for the formulation and optimisation of selenomethionine-loaded chitosan nanoparticles coated with zein for oral delivery. International journal of pharmaceutics, 551(1-2), pp.257-269.
17. Vozza, G., Khalid, M., Byrne, H.J., Ryan, S.M. and Frias, J.M., 2019. Nutraceutical formulation, characterisation, and invitro evaluation of methylselenocysteine and selenocystine using food derived chitosan: zein nanoparticles. Food Research International, 120, pp.295-304.