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Järviö, Natasha

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# Ovalbumin production using *Trichoderma reesei* culture and low carbon energy could mitigate the environmental impacts of chicken egg-derived ovalbumin

Natasha Järviö [1,3*], Tuure Parviainen [2,4], Netta-Leena Maljanen [1,3], Yumi Kobayashi [3,4], Lauri Kujanpää [2], Dilek Ercili-Cura [5], Christopher P. Landowski [2], Toni Ryynänen [1,3], Emilia Nordlund [2], Hanna L. Tuomisto [3,4,6]

[1] Ruralia Institute, Faculty of Agriculture and Forestry, University of Helsinki, Lönnrotinkatu 7, 50100 Mikkeli, Finland

[2] VTT Technical Research Centre of Finland Ltd., P.O. Box 1000, FI-02044 VTT Espoo, Finland

[3] Helsinki Institute of Sustainability Science (HELSUS), University of Helsinki, P.O. Box 4, 00014 University of Helsinki, Finland

[4] Department of Agricultural Sciences, Faculty of Agriculture and Forestry, University of Helsinki, P.O. Box 27, 00014 University of Helsinki, Finland

[5] Solar Foods Oy, Finland

[6] Natural Resources Institute Finland, P.O. Box 2, 00790 Helsinki, Finland

*Corresponding author:

Natasha Järviö

Faculty of Agriculture and Forestry, University of Helsinki, P.O. Box 27, 00014 University of Helsinki, Finland

Natasha.jarvio@helsinki.fi

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**Abstract**

Ovalbumin produced using the fungus *Trichoderma reesei* (Tr-OVA) could become a sustainable replacement for chicken egg white protein powder – a widely-used ingredient in the food industry. Although the approach can generate ovalbumin at pilot-scale, the environmental impacts of industrial scale production have not been explored. Here, we conduct an anticipatory life cycle assessment using data from a pilot study to compare the impacts of Tr-OVA production with an equivalent functional unit of dried chicken egg white produced in Finland, Germany, and Poland. Tr-OVA production reduced most agriculture-associated impacts such as global warming and land use. Increased impacts were mostly related to industrial inputs such as electricity production, but were also associated with glucose consumption. Switching to low carbon energy sources could further reduce environmental impact – demonstrating the potential benefits of cellular agriculture over livestock agriculture for ovalbumin production.

# Main

## INTRODUCTION

The global growing demand for chicken egg white production results in many environmental impacts such as land use, climate change, water scarcity, resource depletion and eutrophication[1–4]. Ovalbumin is the most abundant protein in egg whites, comprising over 50% of egg white proteins. It has been expressed in several host organisms, including *Escherichia coli* and *Pichia pastoris*, mainly on a lab scale[5,6]. Advances in cellular agriculture concepts have made it possible to produce recombinant or cell-cultured ovalbumin on a large enough scale to consider it an economically feasible option to chicken–based egg white powder[7]. Using the filamentous ascomycete fungus *Trichoderma reesei* (*T. reesei*), a well-established and efficient production organism, cell-cultured ovalbumin is now produced in a bioreactor on a pilot-scale. The process is a form of acellular production where the microorganisms are grown to produce an extracellular recombinant protein, in this case ovalbumin (length: 386 amino acids)[6,8]. The coding gene in chickens (*Gallus gallus domesticus*) is SERPINB14[9]. The final product of cell-based production is a protein powder that typically shows comparable functional properties to chicken egg white protein powder and can be used as a replacement in food formulations.

The purpose of this study was to assess the environmental impacts of cell-cultured ovalbumin production in comparison to chicken–based egg white protein powder (hereafter simply referred to as egg white powder, unless otherwise specified) production using an anticipatory life cycle assessment (LCA) method[10,11]. Using an LCA quantifies the environmental impact of *T. reesei*–produced ovalbumin production throughout all production steps and allows for the trade-off comparison between different impact categories[12,13]. The impacts of the production process were estimated for that of an industrial level of 100,000 kg, using data from a pilot production scale and a techno-economic assessment (TEA) produced by VTT[7]. Uncertainties were calculated using Monte Carlo (MC) analysis, while sensitivities of the results were estimated with various sensitivity analyses. Since the production of *T. reesei*–produced ovalbumin mainly relies on the provision of electricity and the carbon intensity of countries varies[14], we also assess the production of *T. reesei*–produced ovalbumin —from now on simply referred to as Tr-OVA — in various countries. The process flowchart in Figure 1 shows the assumed process steps including the most significant inputs and outputs and indicates the main focus on this study.

## RESULTS

### Impact of Tr-OVA for different scenarios

Figure 2 shows the environmental impact of Tr-OVA production per kg of product and contribution per process for four scenarios — Finland, Germany, Poland, and Finland using the low carbon electricity mix that includes both renewable energy sources and nuclear power (SI2 shows the full inputs of this model) — that were chosen to reflect different carbon-intensity levels of country's electricity mixes within the EU[14].

The largest contributor for most impact categories comes from the input of glucose with a share of 2–94%, depending on the impact category and country. For land use, the contribution of glucose most clearly dominates (86–92%), illustrating the reliance of land use of agricultural products. In addition, for water scarcity —also considered a relevant impact category for agricultural products[4]— glucose had a contribution of 58–65%. The second largest contributor to water scarcity is the industrially produced salt mix (22–25%). However, the overall weight of the salt mix (0.85 kg/kg of product) was also 63% lower than the glucose inputs (2.34 kg/kg of product) per kg of Tr-OVA. The antifoaming agent had an overall minor contribution. An exception to this was the contribution of the agent to stratospheric ozone depletion with a range of 81-84%, depending on the scenario.

The differences in the country-specific results are partly explained by the different electricity mixes for each country, where the Finnish electricity mix is dominated by nuclear power (29.1%) and has a high contribution from renewable energy (17.9%)[15], whereas Poland relies mostly on coal (72%)[16]. For example, the total contribution of electricity to global warming potential (GWP) is 34% using the Polish mix but just 2% in the low carbon scenario in Finland. The impacts for freshwater eutrophication and human carcinogenic and non-carcinogenic toxicity show a similar pattern. Results for ionizing radiation, on the other hand, is lowest in Poland. The results clearly show an overall reduction in the environmental impact when producing Tr-OVA using the Finnish low carbon electricity mix. An exception to this is the ionizing radiation, which is explained by the heavy reliance on nuclear power (55.5%) in this particular mix.

## Comparison of Tr-OVA with egg white powder

The calculated *p*-value with the dependent modified null hypothesis significance testing (NHST) led to the rejection of the null hypothesis for all alternatives and impact categories, meaning that the impact of Tr-OVA and egg white powder were significantly different from each other (SI2 contains more information on the statistical test). However, the *p*-value of human carcinogenic toxicity for the comparison of the German alternatives was 0.046, meaning that the result would not have been significantly different at a lower $\alpha$.

Figure 3 shows the deterministic results of our comparison between Tr-OVA produced in Germany and Poland, and egg white powder produced in the respective countries per kg of protein. The results show that for most impact categories typically used for agricultural products (GWP, land use, water scarcity impact, terrestrial acidification and eutrophication potentials), Tr-OVA generally resulted in lower environmental impacts, with the exception of freshwater eutrophication and water scarcity impacts when produced in Poland. For example, the discernibility results showed that 91% and 97% of the MC runs of Tr-OVA production for freshwater eutrophication were larger than of egg white powder, for Germany and Poland, respectively. However, there seems to be a trade-off; for some impact categories more typically burdened by industrial products (ionizing radiation, and human carcinogenic and non-carcinogenic toxicity), the impact of Tr-OVA is higher than that of egg white powder. An exception were the results for ionizing radiation in Poland, where only 49% of the MC runs for Tr-OVA were larger. This partial shift in the environmental burden from the typical agricultural impacts to those impacts typically caused by industry could be explained by the high reliance on industrial processes for Tr-OVA production on the one hand and the agricultural inputs for egg production on the other hand. One example of the high reliance of industrial inputs for Tr-OVA production is the salt mix that has a high overall contribution ranging from 0.3% to 50.5% depending on the impact category. Most of the impact is almost completely attributed by the input of monopotassium phosphate, which made up 41% of the total salt mix by weight. However, monopotassium phosphate was modeled using sodium phosphate as a proxy due to data availability limitations, making the results for the contribution of the salt mix uncertain.

The significantly lower reliance on land for Tr-OVA in comparison to the production of egg white powder using chicken eggs — the discernibility results even show that 100% of the MC runs resulted in lower land use requirements — can be explained by the difference in the total required agricultural resources per kg of protein. According to the World Food LCA Database (WFLDB) by Quantis and Agroscope, chickens require 2.4 kg of feed per kilogram of egg[17]. This means that the feed requirements per kg of protein are 27.5 kg, considering the amount of eggs required and the protein content of egg white produced with eggs. The production of Tr-OVA, on the other hand, requires only 2.54 kg of glucose per kg of protein, supplied with

2.04 kg of minerals and nitrogen. The production of egg white powder using *T. reesei* therefore has a greater agricultural material efficiency in the transformation process of agricultural products to egg white powder than when using chickens.

Although the results of the discernibility test overall show a similar direction in the results for the production of both alternatives in Germany and Poland, the outcome for water scarcity is very different. The results for Germany show that 100% of the runs for egg white powder are larger than that of Tr-OVA, while in Poland 99% of the runs were larger for Tr-OVA per kg of protein. Most of this seems to be caused by a difference in the impact of feed production to water scarcity between Poland and Germany. In the WFLDB model, feed inputs for German eggs are modeled using a generic European average mix in which corn produced in Spain causes 93.1% of the water scarcity impact for egg white powder. In the Polish model, chickens are fed mainly with grains originating from Poland. The water scarcity impact factors for Poland and Spain are very different, namely 1.962 and 77.7, respectively. The difference in these water scarcity-impact factors explain most of the differences between the Polish and German egg white powder results. This difference in results highlights the need for more specific inputs for the German egg production to make conclusions that are more reliable on the impact of Tr-OVA production versus egg white powder for water scarcity.

Although both the production of Tr-OVA and egg white powder require cleaning-in-place (CIP), Figure 3 shows that the environmental impact of CIP for the former is 0.7–106 times that of the latter, depending on the impact category and country. This is partly explained by the use of bioreactors for Tr-OVA production that require regular cleaning.

Despite limitations to our model of the processing of eggs to egg white powder, our results show that the overall contribution of the processing of the eggs are minor compared to egg production, with a total contribution of 0.1–22% for egg processing depending on the impact category and country. This means that the assumptions relating to egg production itself are more important, as shown by the large difference between the impacts resulting from egg production in Germany versus Poland. Limitations of the egg white powder model were mostly related to a lack of land use requirements for the processing of eggs to powder in the original study[18] and the replacement of chlorodifluoromethane with ammonia for cooling in egg white powder production that was due to compliance with EU regulation[19]. This replacement lowered the overall GWP of egg white powder.

## Sensitivity analyses of the Tr-OVA model results

The sensitivity of our results were tested by varying the most relevant inputs of the Finnish model — for example, by increasing one particular input by 20%, changing the background dataset for glucose production, or replacing natural gas in the drying step with electricity. Doing so allowed us to identify which changes in inputs resulted in most variations of the results and to what extent (more background on the changed parameters of the model can be found in Supplementary Table 2). Figure 4 shows the results of the sensitivity analyses in kg per product. There is relatively limited variation in the results for most of the sensitivity tests meaning that most changes in inputs had a minimal effect on the overall estimated impact of Tr-OVA production. For example, despite the high contribution of electricity consumption to the overall environmental impact of Tr-OVA production, an assumed 20% increase in electricity only increased the environmental impact by 0.2–10.9%, depending on the impact category. Two of the sensitivity tests, however, did show a larger effect on the results. The first one was cause by a change of the background database used to model glucose production from the WFLDB used in the original FI scenario to the ecoinvent database used in the sensitivity test named 'FI - Ecoinvent glucose'. The differences in results were most noticeable for land use and terrestrial acidification. Although both datasets use corn starch as an input for glucose production, the assumed amounts differ significantly with ecoinvent assuming 0.9 kg of corn starch per kg of glucose, while WFLDB has an input of 3.48 kg of corn starch per kg of glucose. Nevertheless, the GWP of both systems are the same (1.31 kg $CO_2$ eq / kg glucose).

The other notable sensitivity of the results were due to the assumptions relating to the potential use of the waste product, i.e. GMO *T. reesei* fungal biomass containing some 40–60% moisture, as a feed ingredient.

This was analysed considering multiple impact allocation methods. The GMO *T. reesei* fungal biomass is not yet approved in the EU for feed use; it was thus considered as bio-waste in the main scenarios at this stage. This is likely to change in the future since other by-products from the food and beverage industry are currently used as feed. This is the case, for example, for brewer's yeast; a widely used by-product from the fermentation of beer.

The valorisation of fungal biomass — as opposed to treating it as waste — reduced the overall environmental impact of Tr-OVA production in two ways: the reduction of waste for bio-waste treatment and the sharing of the burden among products. The results in Figure 4 show how the choice of allocation based on a physical relation or an economic relation led to different outcomes for the environmental impact of Tr-OVA production. The protein-based allocation method resulted in a 33.8–41.2% decrease of impacts, depending on the impact category, for Tr-OVA production compared to a 5.5–15.9% decrease using the minimum product sales price (MPSP)-based allocation. The large decrease in the environmental impact of Tr-OVA production is explained by the relatively large amount of fungi mass that contains 45% protein and resulted in a 33.8% allocation factor for a product that was originally considered as waste of the system. We would therefore argue that the MPSP allocation would be the preferred allocation method over the protein-based allocation. The main argumentation for this is that whether or not the waste fungi-mass is used does not affect the decision to produce Tr-OVA or not. Its use would rather be an additional benefit that could improve the environmental impact of Tr-OVA production by reducing the need for waste treatment. This relationship is therefore better reflected using the MPSP as a basis since it results in a higher allocation factor for Tr-OVA (94.6% compared to 66.2% with protein-based allocation). This is also reflected in the preferred use of economic allocation for other agricultural waste-products, such as manure[20,21].

# DISCUSSION

The anticipatory life cycle assessment of cell-cultured egg white protein suggested that production of egg ovalbumin using *Trichoderma reesei* as a host organism instead of chickens could reduce environmental impacts over a range of different impact categories, such as GWP, land use, marine eutrophication, terrestrial acidification, and stratospheric ozone depletion. Most impacts and trade-offs between impact categories could potentially be further reduced using a low carbon energy source. Using alternative and possible waste-sources, such as forestry waste, straw, or cereal side streams, instead of corn-based glucose could potentially further reduce the environmental impact of Tr-OVA production[7]. However, both due to data-availability issues on the production process and an increased level of uncertainty, this could not be further explored within the scope of this article. For example, the use of lignocellulosic side-streams requires additional process steps, such as pre-processing by steam explosion or diluted acid hydrolysis, which are processes that are yet to be used in food production.  As glucose using corn starch was identified as one of the main contributors to the environmental impact of Tr-OVA in this present study, we encourage future research to explore these possibilities.

The uncertainty of the results remains high since the process is not yet in industrial operation. For example, the purification step of Tr-OVA has not yet been tested on a commercial scale. Other uncertainties were caused by the lack of life cycle inventory data on some inputs, such as monopotassium phosphate, and the lack of more accurate information on CIP requirements. We tried to capture most uncertainties and sensitivities of the model with the use of high uncertainty ranges and the use of a sensitivity analysis. This increased the robustness of the results over the range of different scenarios. The results therefore provided a good initial overview of the possible ranges within which the impact of Tr-OVA production would likely fall, and how these related to the production of egg white powder. Additionally — although not peer-reviewed — similar results for non-allocated GWP were found in a recent report by Perfect Day on the production of animal-free whey protein containing 90% protein and using the same host organism, *T. reesei,* for its production process, in the United States (US).

Nonetheless, more attention to practical measurements in industrial production is required to improve the accuracy of the results from an anticipatory study to commercial process lifecycle assessment in the future. As identified by the sensitivity test, a relevant modelling choice for future research would be the potential to use its by-products in the future for feed production or other added-value applications. Additionally, we were able to identify the impact of database choices and quality on the results for both Tr-OVA and egg white powder and would recommend further development and accuracy of product systems in the different databases.

# Methods

## Goal and scope of the LCA study

The goal of this study was to estimate the environmental impacts of industrial-scale production of ovalbumin synthesised by *T. reesei*. We applied an anticipatory LCA with a cradle-to-gate system boundary, based on current data gathered and estimated from a functioning pilot production scale. Additionally, we used a TEA of Tr-OVA production that was performed to assess the process engineering requirements and device capabilities[7]. TEA results were used to find significant steps in the production chain that would influence the environmental load[7].

The environmental analysis of Tr-OVA production was modeled using the Simapro 9.1.0.11 PhD software package[22] using the ecoinvent 3.6 database. We used the ReCiPe 2016 Midpoint (H) method to calculate the global warming potential (GWP, kg $CO_2$ eq), land use ($m^2$a crop eq), freshwater and marine eutrophication potential (kg P-eq; kg N-eq), terrestrial acidification (kg $SO_2$ eq), ionizing radiation (kBq Co-60 eq), human carcinogenic and non-carcinogenic toxicity (kg 1,4-DCB; kg 1,4-DCB), and stratospheric ozone depletion (kg CFC11 eq)[23]. Water scarcity was assessed using the AWARE method[24]. Because the production of Tr-OVA is an industrial food manufacturing process relying on electricity and natural gas, we included impact categories that are commonly used for both agricultural and industrial food manufacturing LCA studies[18,25–28]. Because of the industrial nature of the product, the life cycle industrial energy use was also assessed using the cumulative energy demand (CED) V1.1 method by ecoinvent[29].

Two functional units (FU) were used in this study. The first FU is expressed as 1 kg of Tr-OVA product with an 8% moisture content and a 92% protein content and serves to reflect the environmental impact of the product. The second FU used is that of 1 kg of protein. As Tr-OVA is a drop-in substitute that can replace protein from egg white powder[7], the second FU is used to compare the environmental impacts of both products. The cradle-to-gate system boundaries of this model start at the extraction of raw materials, includes the production of Tr-OVA and the cleaning of the facilities, and ends at the factory gate. The flowchart of the system is shown in

RESULTS . Excluded were the inoculum preparation phase, packaging and the materials and construction of facilities. Land use for facilities, however, was included in the model.

## System description

The production of Tr-OVA starts with the cultivation of fungal spores of engineered fungus *T. reesei* at 28°C. The process then moves on to the pre-culture of the strain. This is a three-stage process where the fungi are fed with a continuous supply of water mixed with chemicals and nutrients for growth at 28°C. After that, the mycelium is harvested with a two-stage process performed at 28°C and inoculated in a bioreactor where fermentation will take place. During fermentation, the *T. reesei* fungus is supplied with glucose as the carbon source and other nutrients that are needed for growth in the fermentation process (Supplementary Table 1). Because the fermentation process produces heat, the fermented suspension needs to be cooled, sparged, and mixed throughout the process. An assumed production of 100,000 kg Tr-

OVA requires the use of 5 bioreactors for cultivation in the sizes of 0.06, 0.6, 9, 63, and 125 $m^3$. These bioreactors are cleaned using the cleaning-in-place (CIP) method after each fermentation cycle, which amounts to an estimated 50 cleaning operations per year.

After fermentation, the growth media moves on to the filter press where the fungal biomass (solids) is separated from the produced proteins (liquid). This rejected fungal biomass leaves the system with a 58.3% moisture level. The filtrate with ovalbumin protein moves on to an ultrafiltration step, where 35.6 kg water per kg ovalbumin product is removed as permeate. The retentate then enters the spray-drying phase where it is heated and dried to generate an end product in a powder format that is ready to be packed. The fermentation process was tested on a pilot scale at VTT during 2018–2019. The main fermentation parameters, such as feedstock and fermentation temperatures, were based on these test results. Energy consumption and mass flows were based on modeling[7]. During the verification of the model, the process was compared to the most similar existing processes, such as the NREL's *T. reesei* process[30].

## Scenarios

Industrial fermentation processes use significant amounts of energy, thus we decided to create four different production scenarios based on different production locations. We compared Tr-OVA production using the average electricity mix of Finland, Germany, and Poland. The locations were selected based on the stepwise levels of carbon intensity per kWh. In Finland, the carbon intensity is 204 g C $kWh^{-1}$, Germany 588 g C $kWh^{-1}$ and Poland 911 g C $kWh^{-1}$ [14]. In addition, we created a scenario using a low carbon intensity electricity mix within Finland, which consists of non-combustion–based energy technologies[31]. This electricity mix was modeled conserving the ratios of the low carbon energy sources listed in the original Finnish electricity mix based on data provided in the ecoivent database. Low carbon electricity has a carbon intensity of less than 50 g C $kWh^{-1}$ [31].

Water use was modeled by adjusting the ecoinvent tap water process for Europe without Switzerland. In Finland, 65% of tap water is extracted from groundwater sources[32]. We assumed that the rest of the tap water was sourced from lakes[33]. Tap water in Poland mostly comes from surface water (75%) and 25% is from groundwater[34]. Groundwater is the most important water source of Germany, providing more than 69% of the delivered tap water, while 15% comes from surface water and the remaining 16% from other resources such as artificially recharged groundwater[35].

## Data collection

The assessment of the environmental impact of Tr-OVA production on an industrial scale was based on the pilot production scale and the TEA produced by VTT[7]. Our LCA model was based on the input and output requirements of the pilot production and scaled to an industrial production level with an assumed 100,000 kg annual output. For more information on the model behind the assumed inputs and outputs required for industrial production level, we refer to the article and supportive information by Voutilainen et al.[7]. Supplementary Table 1 provides an overview of all inputs and outputs of the system per FU. The production of Tr-OVA on an industrial scale would use standard industrial fermentation and some down-stream-processing (DSP) machinery that are used in large-scale production of single-cell proteins such as the Quorn process[36]. A major difference in DSP is the separation phase, since the ovalbumin needs to be purified from the *T. reesei* biomass, other co-produced proteins and growth media.

The utility requirements, including steam, electricity, chemicals, and process water, were based on material and energy balance calculations. Due to limitations in the ecoinvent database, some nutrient inputs of the system were modeled using a proxy. These proxies were selected based on experts' opinions on similarities of properties or functions. The use of natural gas in the spray dryer was modeled by adjusting the market for low-pressure natural gas from the ecoinvent database to the country-specific natural gas mix. The emissions from combustion of natural gas were modeled following the guidelines and emission factors published by the International Panel on Climate Change (IPCC)[37,38].

Direct land use requirements were roughly estimated to be 1000 $m^2$ for all facilities based on the assumed production scale and were modeled as land occupation[7]. We assumed that the factory would be in operation for about 20 years, meaning that the transformation of 1000 $m^2$ were allocated over 2,000,000 kg of Tr-OVA. See Supplementary Table 1 for details.

Waste coming from the system is mainly in the form of fungus mass, with a 40–60% moisture level, and wastewater from CIP. We assumed that there was no wastewater flow from the production process itself since all water was released as evaporated water into the air during the spray-drying phase. Fungus mass was assumed to be treated as biowaste in a biowaste treatment facility. SI1 provides details on the exact assumptions behind this part of the model.

The CIP requirements were estimated based on the water and detergent requirements for the typical cleaning of bioreactors used in industrial-scale food production. We assumed a CIP system that uses a partial re-use system in which water and detergent requirements are reduced[39]. The electricity requirements, as well as the emissions related to effluent of CIP, were estimated using the article by Eide et al.[40] on CIP methods for dairies. This was decided on the basis that both the production of Tr-OVA and milk result in proteinaceous deposits.

Treatment of wastewater from CIP of the five bioreactors was modeled using the process of average wastewater treatment in Europe without Switzerland from the ecoinvent database. Additionally, we conservatively assumed that the treated water did not return to the original source and ecosystem of water abstraction. This is, for example, the case of wastewater treated in the Helsinki area, in Finland[41]. Additionally, this avoids the results of potential negative numbers for water scarcity (this has to do mathematics behind the models calculations and was further explained in Järviö et al.[42]). We, therefore, adjusted the original ecoinvent process so that any water outputs (i.e. representing the return of water to its original source) were set to zero. SI1 provides details on the exact assumptions behind this part of the model.

## Comparison to egg white production

The results of the environmental impacts of Tr-OVA production were compared to that of egg white powder production. We used the inventory data published in an article by Tsai et al.[18] on the production of egg yolk powder including CIP using the continuous flow to remodel the emissions for egg white powder production. However, the moisture content of egg white is much higher than egg yolk, with 88% versus 48%, respectively[43,44]. Where Tsai et al.[18] assumed 2.18 kg of liquid egg yolk per 1 kg of egg yolk powder, we assumed 5 kg of liquid egg white for the production of 1 kg of egg white powder with an 8% moisture content. Combining this data with the input of eggs as per 1 kg of liquid egg white reported by Tsai et al.[18] means that the total amount of eggs needed per 1 kg of egg white powder equals 9.15 kg. Because of the higher moisture content in liquid egg white than egg yolk, we also adjusted the input requirements for the drying step. We assumed that the process of drying egg white would be similar to that of drying Tr-OVA. Because moisture contents of the unfinished wet product before the drying step are quite similar — 12% and 13.3% for liquid egg white and Tr-OVA production, respectively — we used the same inputs per kg product. This meant that the kWh for drying liquid egg white were less than originally listed in the article by Tsai et al.[18]. However, since drying inputs are highly dependent on the assumed efficiency of the system, the comparison of the two products would be fairer when based on the same assumptions.

The emissions resulting from egg production and breaking, storage, and pasteurization (BSP) were allocated based on the mass of the output products, where egg white makes up 55% of all outputs. This was based on the assumption that eggshells and residue are considered to be a by-product of the system[45]. We used data for egg production from the WFLDB by Quantis and Agroscope since it relies on the ecoinvent 3.5 cut-off system in its background model. Egg production for several countries were given, including Germany and Poland but not Finland. We therefore decided to make the comparisons between egg white powder and Tr-OVA for only these two countries. Furthermore, it was assumed that eggs travel about 100 km by truck from the farm to the egg white production plant. See S2 for the full model based on the inventory data of Tsai et al.[18].

We validated our model on egg white powder production by additionally constructing a model for egg yolk powder production using the inventory data given in the article by Tsai et al.[18]. The results of this egg yolk powder model were compared to the results reported by the authors themselves. The GWP results for our model were initially much higher. By far, most of the GWP was caused by the use of chlorodifluoromethane that Tsai et al.[18] reported to be 0.079 kg per 1 kg of egg yolk powder. Because of the discrepancy in results and because chlorodifluoromethane cannot be used as a refrigerant within European Union (EU) countries due to its high ozone-depletion potential and GWP[19], we decided to replace the chlorodifluoromethane with ammonia in our egg white powder–production model. Ammonia is a natural refrigerant that can be used for cooling in commercial refrigeration[46].

One major difference between the ecoinvent database and the WFLDB is that the latter includes the emissions from LUC. As this can be a major source of emissions contribution to the total GWP of food products[1,47], we decided to model glucose in the Tr-OVA production model using the WFLDB. This was to avoid unaligned system boundaries of the two product systems and a subsequent underestimation of the GWP of glucose used in the Tr-OVA production. However, glucose in the WFLDB is modeled at plant. To transform this into an "at market" product, we included the estimated transportation distances used in the ecoinvent database.

Both products were compared based on the protein content using the second FU since the functionality of the end-product is determined by the protein. For example, the proteins are used to form the texture in a cake-making application. Egg white powder contains 79.8% protein[48].

## Uncertainty analysis and statistical tests

The environmental assessment of Tr-OVA production was based on the estimated inputs and outputs for Tr-OVA production on an industrial scale, using gathered and estimated data of Tr-OVA production on a pilot scale. Data uncertainties were high, therefore the uncertainties of the results were analyzed using a Monte Carlo (MC) analysis modeled using the Simapro 9.1.0.11 PhD software package. The result of a MC analysis is a probability distribution within which the results are likely to fall, based on repetitively calculating the environmental impact a number of times[49]. It is a commonly used tool to capture uncertainty within LCA studies[50]. To perform the MC analysis, uncertainties were captured using a uniform distribution of inputs with a ±20% margin for the production of Tr-OVA (SI2 provides more details). Since the article by Tsai et al.[18] did not provide uncertainty ranges, we applied the pedigree method to add uncertainties to the egg white powder. The MC simulation was performed in Simapro using a limited number of 100 iterations[50]. We used a seed value of zero for all MC simulations to simulate dependent sampling. Doing so allowed us to account for common uncertainties between the Tr-OVA and chicken egg–based egg white powder and enabled a statistical comparison of the results[51]. In addition, we applied the parametric bootstrap method to handle the large uncertainty ranges of water scarcity that results from the incorrect estimation of probability distributions of the AWARE characterization factors[52]. We used Python 3.0 to run the bootstrap method, running 1,000 simulations with a sample size of 300 allowing for replacements. Any possible negative values that might naturally result from the MC analysis but are not sensible were ignored from the analysis[53]. Both a discernibility test[54] and the dependent modified null hypothesis significance testing (NHST)[55] were used to both explore the differences in impacts between Tr-OVA and chicken-based egg white powder and to confirm which alternative is significantly different. The dependent modified NHST testing was performed using a significance level $\alpha$ of 0.05 and a difference threshold of $\delta_0$ of 0.2. The null hypothesis is $H_0: S_{i,j,k} \le \delta_0$, where $S$ refers to the standardised difference of means, $i$ and $j$ to the different alternatives and $k$ to the impact. The p-value was calculated using a one-sided (right) cumulative distribution function[54,55]. Both statistical tests were performed on a per kg of protein basis.


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## Competing interests

Author Tuure Parviainen is a co-founder, shareholder and from 20th April 2021 employed by the start-up company Volare Solutions Ltd. (Finland), which aims to commercialize the production of Hermetia illucens L. from industrial side-streams and its use as feed (non-food) protein ingredient. This process however is unrelated to this article. All other authors declare no competing interest.

## Author information

Affiliations

**Ruralia Institute, Faculty of Agriculture and Forestry, University of Helsinki, Lönnrotinkatu 7, 50100 Mikkeli, Finland**

Natasha Järviö, Netta-Leena Maljanen & Toni Ryynänen

**VTT Technical Research Centre of Finland Ltd., P.O. Box 1000, FI-02044 VTT Espoo, Finland**

Tuure Parviainen, Lauri Kujanpää, Christopher P. Landowski, Emilia Nordlund

**Helsinki Institute of Sustainability Science (HELSUS), University of Helsinki, P.O. Box 4, 00014 University of Helsinki, Finland**

Natasha Järviö, Netta-Leena Maljanen, Yumi Kobayashi, Toni Ryynänen, Hanna L. Tuomisto

**Department of Agricultural Sciences, Faculty of Agriculture and Forestry, University of Helsinki, P.O. Box 27, 00014 University of Helsinki, Finland**

Tuure Parviainen, Yumi Kobayashi, Hanna L. Tuomisto

**Solar Foods Oy, Finland**

Dilek Ercili-Cura

**Natural Resources Institute Finland, P.O. Box 2, 00790 Helsinki, Finland**

Hanna L. Tuomisto

## Contributions

N.J, T.P, N.L.M., Y.K, C.P.L, E.N, and H.L.T designed the work, N.J, T.P, N.L.M., L.K. C.P.L, E.N, and H.L.T collected data, N.J, T.P., N.L.M. created the model, N.J. and T.P performed the interpretation and drafted the manuscript with valuable inputs from N.L.M., Y.K., L.K., D.E.C., C.P.L., T.R, E.N., and H.L.T. All authors reviewed and approved the final manuscript.

## Corresponding author

Correspondence to Natasha Järviö.

## Figure Legends/Captions (for main text figures)

**Figure 1 title:** System diagram of the processes involved in the production of ovalbumin produced by *T. reesei* (Tr-OVA).

**Figure 2 title**: Environmental impact of Tr-OVA production per scenario

***Figure 2 legend**: Deterministic results and process contributions in Finland (FI), Germany (DE), Poland (PL) and Finland using the low carbon electricity mix (FI-LC) per kg of Tr-OVA product. Standard deviations (s) from the Monte Carlo runs (n=100) are indicated with a black line. Note: CFP refers to cultivation, filtration, and purification and Tr-OVA production refers to direct emissions and land use caused by the Tr-OVA production system.*

**Figure 3 title**: Comparison of the environmental impact of Tr-OVA with egg white powder

***Figure 3 legend**: Deterministic results and process contributions for the production of Tr-OVA in Germany (DE) and Poland (PL) versus egg white powder production using chicken eggs in Germany (DE) and Poland (PL) per kg of protein. Standard deviation (s)* *from the Monte Carlo runs (n=100) are indicated with black lines. Note: BSP refers to breaking, storage, and pasteurization and CFP to cultivation, filtration, and purification.*

**Figure 4 title**: Sensitivity analyses of the Finnish Tr-OVA model per kg of Tr-OVA product.

***Figure 4 legend****: Results of the sensitivity analyses based on changed inputs of the Finland (FI) scenario, per 1 kg of Tr-OVA product. Tested were a change of input of natural gas to electricity for the drying step (FI – electric drying); An 20% increase of the main inputs (electricity, natural gas, ammonia); change in the database use of glucose production (FI – Ecoinvent glucose); and an allocation of environmental impact to by-products (fungal biomass) based on protein content or minimum product sales price (MPSP) (FI - By-product). Results of the Monte Carlo (MC) runs (n=100), to estimate the uncertainties of the analyses, are displayed using a box-and-whisker plot to indicate the $0^{th}$, $25^{th}$, $50^{th}$, $75^{th}$ and $100^{th}$ percentiles, dots indicate outliers and deterministic results of the sensitivity analyses are shown using circles.*

# Data availability

The authors declare that to the best of our ability we have provided the data supporting the findings in this paper and its supplementary information files. Any additional data, particularly related to adjustments made in the background processes of our model, are available upon request from the corresponding author.

# Code availability

Code that was used to generate results for this study are freely available upon request from the corresponding author.

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