**School of Population Health**

# The effect of UV radiation, cooking and storage on the D vitamer content of dried *Agaricus bisporus* mushrooms

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**This thesis is presented for the Degree of Doctor of Philosophy of Curtin University**
**School of Population Health**

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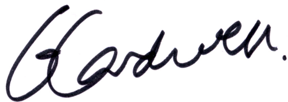
**Declaration**

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.



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Date: 28th February 2023

**Acknowledgement of Country**

*We acknowledge that Curtin University works across hundreds of traditional lands and custodial groups in Australia, and with First Nations people around the globe. We wish to pay our deepest respects to their ancestors and members of their communities, past, present, and to their emerging leaders. Our passion and commitment to work with all Australians and peoples from across the world, including our First Nations peoples are at the core of the work we do, reflective of our institutions' values and commitment to our role as leaders in the Reconciliation space in Australia.*

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Pilot studies were conducted at SA Mushrooms in Adelaide, South Australia using their commercial pulsed light machine. Free access to machine and mushroom samples was generously provided by Managing Director Nick Femia. Samples for the studies were free-of-charge with the kind support of Costa Mushrooms in Perth. Eduardo Herrera-Tapia from White Prince Mushrooms in Sydney was very helpful in loaning their radiometer so that I could conduct calibration trials with the pulsed light machine.

During the thesis, advice and support was provided by Professor Jane Scott before her retirement and Professor Gavin Pereira through until the conclusion. Dr Toni Hannelly helped me navigate a unit titled Health Research Methods, which was required before being accepted to carry out my studies.

I spent many hours in the food laboratory and my thanks go to Dr Syed Nasar Abbas for training me to use a freeze-dryer and Edwin Junaldi for all his help with the data-logger and the

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**Publications, presentations, awards**

*Publications*

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*Poster*

**Cardwell G.**, Black L.J., Bornman J.F, James A.P., Strobel N. Pulsed UV radiation increases the vitamin D concentration of fresh and dried mushrooms in a dose dependent manner. 5th International Vitamin Conference, Sydney 8-10 August 2018 (see Appendix I)
This poster was also a finalist in the Curtin University Research Rumble student poster display (2019)

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*Awards and prizes*

- Winner, People's Choice, 3 Minute Thesis, Curtin University 2017
- Runner-Up, 3 Minute Thesis, Curtin University 2017

**Abstract**

The human body produces vitamin D through the action of ultraviolet-B (UV-B, 280-315 nm) radiation directly on the skin. It is also available from the diet. Unfortunately, most of the population cannot obtain sufficient vitamin D from food alone as common sources are limited to fish, meat, egg yolk and vitamin D-fortified foods such as some milks, margarines and breakfast cereals.

Members of the biological kingdom fungi, such as mushrooms and yeast, can also produce vitamin D. When exposed to a source of UV-B radiation, for example a UV lamp or sunlight, mushrooms convert their abundant pro-vitamin $D_2$ (ergosterol) to vitamin $D_2$ in amounts that can provide the daily vitamin D requirements in a 100 g serving of fresh mushrooms or 20 g serve of dried mushrooms. The most time-efficient manner to exploit this phenomenon is to expose mushrooms to pulsed UV radiation, which produces nutritionally relevant concentrations of vitamin $D_2$ in less than a second. In contrast, to reach similar concentrations of vitamin $D_2$, a conventional UV lamp can take 15-30 minutes depending on lamp intensity, and exposure to solar radiation can take up to an hour depending on season, latitude and weather conditions.

The opening chapter of this thesis introduces the fascinating mushroom and its unique capability to produce D vitamers when it is exposed to a source of UV radiation. Growers in Australia and the United States have already placed some vitamin D-enriched fresh mushrooms on the market, yet there may be more potential for dried or powdered mushrooms as a source of vitamin D due to their long shelf-life. Common button mushrooms (*Agaricus bisporus*) were chosen as this species comprises more than 90% of mushrooms sold in Australia and is one of the three most consumed mushrooms worldwide. If Australian farmers produce dried or powdered vitamin D-enriched mushrooms, they are most likely to use the common button mushroom.

The second chapter is a review of the mushroom as a potential dietary source of vitamin D. This is the first comprehensive review of the topic and documents the influence of sunlight and UV radiation on the natural phenomenon of vitamin D production in mushrooms. There were very few studies of UV-irradiated, hot air-dried mushrooms, either sliced or whole, yet air-drying is the common commercial method of producing dried mushrooms. There were no studies of the ideal sequence of irradiating and drying, or the influence of rehydrating and

cooking dried mushrooms. Although sun-drying mushrooms is common in Asian countries, it may affect the quality of the mushroom, while the amount of vitamin D produced is difficult to predict. Dried mushrooms are very under-researched when considering it is a very popular food source in many Asian countries and exported around the world.

As mushrooms readily generate vitamin D in response to UV exposure, Chapter 3 describes studies investigating whether there is any difference in the production of D vitamers in dried mushrooms depending on the sequence of exposure to UV radiation and drying. It was clear that irradiating mushrooms when they are fresh, prior to air-drying, was more effective at generating D vitamers than irradiating dried mushrooms. This is most likely due to moisture being required for the conversion of ergosterol to vitamin $D_2$, although the reason is not certain. Based on this finding, fresh mushrooms that were first irradiated, then air-dried, were used in the subsequent studies. The same irradiation dose and air-drying parameters were used in the two studies on the influence of storage times and cooking methods on the retention of D vitamers.

In Chapter 4 the effect of storage on the retention of D vitamers was explored. Most dried mushrooms are imported and sold at specialty shops, whereupon they are purchased and taken home to be stored for some weeks and possibly months. Although dried mushrooms can contain plentiful vitamin $D_2$, it is important to know the rate of degradation, if any, of this vitamin over time under common storage conditions. Fresh mushrooms were irradiated with the same dose as in the experiment in Chapter 3, then air-dried and vacuum sealed in plastic pouches as occurs for commercial dried mushrooms. The concentrations of D vitamers were then analysed following 3, 6 and 12 months of storage. It was found that most of the loss of D vitamers occurred in the second six months of storage with 58% of vitamin $D_2$ and 68% of 25(OH)$D_2$ being retained at 12 months.

Chapter 5 addressed the effect of cooking on the retention of D vitamers in UV-irradiated, dried mushrooms. It is important to know how much of the D vitamers are retained after common methods of cooking. There would be little value in producing vitamin D-enriched dried mushrooms if most of the D vitamers are lost during cooking. This study revealed that there was excellent retention after cooking with ≥95% of the D-vitamers being retained after frying, baking and boiling.

This thesis has established the best sequence of irradiation and drying of mushrooms for the generation of D vitamers, confirmed the stability of these vitamers over 12 months of household storage, and confirmed the retention of most of the D vitamers following common rehydration and cooking methods. Hence, consumption of UV-irradiated dried mushrooms are a potential low cost means of improving the vitamin D status in vulnerable communities, especially in those consumers who choose to avoid animal sources of vitamin D.

**Statement of contribution**

My contribution to each of the included publications and manuscripts has been detailed and endorsed by co-authors (Appendix IV).

| **Publication** | **My contribution** |
| --- | --- |
| A review of mushrooms as a potential source of dietary vitamin D | Reviewed and selected the papers; wrote the original draft with the guidance of co-authors Associate Professor Lucinda Black, Dr Anthony James and Professor Janet Bornman; wrote the responses to reviewers' comments with co-authors' suggestions. The review was published in the journal *Nutrients* (Impact Factor 6.7; Q1 Food Science). |
| Effect of air-drying on the generation of vitamin $D_2$ and 25-hydroxyvitamin $D_2$ by pulsed UV irradiation in button mushroom (*Agaricus bisporus*) | Procured the pulsed light machine and radiometer.<br>Designed the study, obtained mushroom samples, irradiated samples, dehydrated samples and measured D vitamers.<br>Analysed samples for the pilot studies, while the samples for this study were analysed by staff at the National Measurement Institute (NMI) due to COVID-19 travel restrictions.<br>Wrote the original draft for the *Journal of Food Composition and Analysis* (Impact Factor 4.5; Q1 Food Science). Wrote the original responses to the reviewer comments and compiled the final manuscript and reviewer responses, with co-author suggestions.<br>Under the supervision of chemist Norbert Strobel and Dr. Saman Buddhadasa at the NMI, I analysed D vitamers in mushrooms (vitamin $D_2$, 25-hydroxyvitamin $D_2$, vitamin $D_3$, 25-hydroxyvitamin $D_3$ and vitamin $D_4$).<br>The method was subsequently verified for a mushroom matrix in April 2019 based on the National Association of Testing Authorities method VL454 (ISO17025:2017) approved in 2017. The study was published in the *Journal* |

| | |
|---|---|
| | *of Food Composition and Analysis* (Impact Factor 4.5; Q1 Food Science)<br>Although I did the laboratory analysis, all credit for assistance and refinement of the method goes to the staff at NMI. |
| Vitamin $D_2$ and 25-hydroxyvitamin $D_2$ retention in pulse UV-irradiated dried button mushrooms (*Agaricus bisporus*) after 3, 6 and 12 months of storage | Designed the study with the assistance of supervisors. Selected and obtained mushroom samples direct from the farm. Conducted the irradiation, dehydration, storage and arranged the analysis of D vitamers by NMI.<br>Wrote the original manuscript, with the valuable assistance of the co-authors, for submission to the journal *Foods* (Submitted 8 February 2023; Impact Factor 5.7; Q1 Food Science). |
| Effect of household cooking on the retention of vitamin $D_2$ and 25-hydroxyvitamin $D_2$ in pulse UV-irradiated, air-dried button mushrooms (*Agaricus bisporus*) | Designed the study with the assistance of supervisors and co-authors. Selected and obtained mushroom samples, followed by irradiating, air-drying, rehydrating, cooking, and measuring all outcomes.<br>Measured all weights, temperatures and water pH.<br>Wrote the original manuscript, with the valuable assistance of the co-authors, for submission to the journal *Food Chemistry* (Submitted 27 January 2023; Impact Factor 9.2; Q1 Food Science; Q1 Analytical Chemistry). |

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**List of abbreviations**

| | |
|---|---|
| $25(OH)D_2$ | 25-hydroxyvitamin $D_2$ |
| DW | Dry weight |
| EFSA | European Food Safety Authority |
| FW | Fresh weight |
| kJ | Kilojoules |
| LC-QQQ | Liquid chromatography with triple quadrupole tandem mass spectrometry |
| LOD | Limit of Detection |
| LOQ | Limit of Quantitation |
| nm | Nanometres |
| NATA | National Association of Testing Authorities, Australia |
| NH&MRC | National Health & Medical Research Council |
| NMI | National Measurement Institute, Melbourne, Victoria, Australia |
| SD | Standard Deviation |
| UV | Ultraviolet |

## Thesis outline

This thesis comprises an introduction to mushrooms and vitamin D (Chapter 1), a published literature review (Chapter 2), three original research chapters (Chapters 3-5) and a discussion (Chapter 6).

## Chapter 1: Background

Vitamin D is an ancient vitamin, evolving at least 500 million years ago. It is well known that humans can produce vitamin D through exposing skin to sunlight. Less understood is that members of the fungi family, such as edible mushrooms and yeast, also produce vitamin D when exposed to a source of UV radiation, whether from sunlight or a UV lamp. Hence, wild mushrooms produce vitamin D, as do mushrooms that are dried in the sun, a common method of producing dried mushrooms in China. Domestic mushrooms available from supermarkets are generally low in vitamin D as they are grown in environment-controlled growing rooms where the only light is from fluorescent (non-UV) light. If farmers grow mushrooms under a UV lamp, or expose them to UV radiation post-harvest, the mushrooms will produce nutritionally relevant amounts of vitamin D. Fresh mushrooms, dried mushrooms and even powdered mushrooms will all generate vitamin D if they receive sufficient UV radiation. As the dietary sources of vitamin D are limited to fish, eggs, some meats and a small number of vitamin D-fortified foods, UV-exposed mushrooms are a viable, non-animal source of vitamin D. If the mushrooms are dried, they then become a long shelf-life source of vitamin D.

## Chapter 2: A review of mushrooms as a potential source of dietary vitamin D

A literature review was conducted and published in the journal *Nutrients* in October 2018. It was the first comprehensive review to collate all the research on the generation of vitamin D in edible mushrooms (>100 citations, Scopus). Since the review there have been other published papers in this field. They have been summarised at the end of this chapter. Most of the research continues to be on fresh edible mushrooms. The review identified gaps in the literature regarding the D vitamer concentration in dried mushrooms.

## Chapter 3: Effect of air-drying on the generation of vitamin $D_2$ and 25-hydroxyvitamin $D_2$ by pulsed UV irradiation in button mushroom (*Agaricus bisporus*)

Previous research has shown that fresh, dried and powdered UV-exposed mushrooms all have the capability to produce vitamin $D_2$ in a dose dependent manner. There is very little research on dried mushrooms. It was unclear if the generation of D vitamers was more efficient when

exposing mushrooms to UV radiation before or after drying, or if there was no difference. At the UV dose selected (which was based on a previous pilot study), it was more efficient to expose mushrooms prior to, rather than after, air-drying. UV-exposed fresh mushrooms generated both vitamin $D_2$ and 25(OH)$D_2$, while UV-exposed dried mushrooms produced lesser amounts of vitamin $D_2$ and, surprisingly, no 25(OH)$D_2$ at all. It is possible that air-drying before UV exposure had denatured the cytochrome P450 enzyme that produces 25(OH)$D_2$ or there was insufficient moisture present for the process to occur. Nevertheless, exposing mushrooms to UV radiation before air-drying is a more efficient manner to generate D vitamers and this information will help mushroom farmers who want to produce dried and powdered vitamin D-enriched mushrooms.

**Chapter 4: Vitamin $D_2$ and 25-hydroxyvitamin $D_2$ retention in pulse UV-irradiated dried button mushrooms (*Agaricus bisporus*) after 3, 6 and 12 months of storage**

Pulse UV-exposed, then air-dried mushrooms were stored for 0, 3, 6 and 12 months under conditions to replicate commercial packaging and household storage. Typically, dried mushrooms would be consumed within 12 months of harvesting and packaging. As expected, the concentration of D vitamers declined over time. After 12 months of storage there still was sufficient D vitamers to provide more than the recommended daily needs in a 20 g serving of dried mushrooms. This was the first study of retention of D vitamers in dried mushrooms using pulsed UV radiation and analysis of 25(OH)$D_2$.

**Chapter 5: Effect of household cooking on the retention of vitamin $D_2$ and 25-hydroxyvitamin $D_2$ in pulse UV-irradiated, air-dried button mushrooms (*Agaricus bisporus*)**

This was the first study on the retention of D vitamers after the cooking of UV-irradiated dried mushrooms. At the time of the study, there were two studies on the retention of D vitamers after cooking fresh, UV-irradiated mushrooms and each of them had shown a generally high retention of vitamin $D_2$. It was quite plausible that dried mushrooms, due to the cellular destruction caused by air-drying, would retain only small amounts of D vitamers, with losses during rehydration and cooking. However, the study demonstrated that dried mushrooms that were rehydrated and then cooked retained at least 95% of their D vitamers when compared to the rehydrated mushrooms (controls). Therefore, if regulations permit it, manufacturers of dried, vitamin D-enriched mushrooms could promote the vitamin D content on the packaging.

**Chapter 6: Discussion**

The final chapter discusses the research outcomes and their potential to be used for the commercial production of vitamin D-enriched dried mushrooms and their storage and use by the consumer, especially in those communities with limited access to refrigeration and those preferring a non-animal source of vitamin D. The studies are complemented by other published human research confirming the bioavailability of vitamin $D_2$ following the consumption of UV-irradiated mushrooms. In addition, animal studies show that mushroom-derived vitamin $D_2$ has the potential to augment bone health. The chapter also highlights the strengths and some shortfalls in the thesis studies, and some gaps worth pursuing through future research. It concludes with a summary of the thesis.

## Chapter 1: Background

### 1.1 The biological kingdom of mushrooms

*In the course of forty years practice and observation, I have generally remarked, that the culture of the Garden Mushroom has proved considerably more precarious and unsuccessful than that of any other kitchen-garden vegetable; or even of almost any other cultivated plant of our gardens; and that its true nature is little known among the generality of gardners (sic).*

*This plant is of so very singular a growth and temperature, that, unless a proper idea of its nature and habit is attained, and the peculiar methods and precautions pursued in the process of its propagation, little success will ensue. The whole management of it remarkably differs from that of every other species of the vegetable kingdom; and it is the most liable of any to fail, without a very strict observance and care in the different stages of its cultivation.*

John Abercrombie, from his book *The Garden Mushroom: its Nature and Cultivation.* (Abercrombie, 1779)

Abercrombie, in possibly the first English language book on mushroom cultivation, had recognised the difficulty in growing garden mushrooms (*Agaricus campestris*), very similar to the cultivated mushroom that is the subject of my studies (*Agaricus bisporus*). What Abercrombie did not know at the time was that mushrooms were not vegetables but fungi, and they belonged in their own biological kingdom.

Lifeforms are commonly classified into five distinct biological kingdoms – Monera (e.g. bacteria), Protista (e.g. seaweeds and algae), Plantae (e.g. grains, fruits and vegetables), Fungi (e.g. mushrooms and yeast), and Animalia (e.g. insects and mammals) (Margulis & Chapman, 2010). Each kingdom evolved at a different time as their cellular complexity increased (Fig. 1).

In fact, mushrooms are more closely related to animals than plants. For example, the cell wall rigidity of mushrooms is provided by the polysaccharide chitin, which also provides the strength to the exoskeletons of arthropods and insects (Vetter, 2007), while the cell wall structure in plants comes mainly from the polysaccharide cellulose (Keegstra, 2010).

Mushrooms are different to other foods we eat, and one specific difference is their capability to generate vitamin D, the characteristic which is the topic of this thesis.

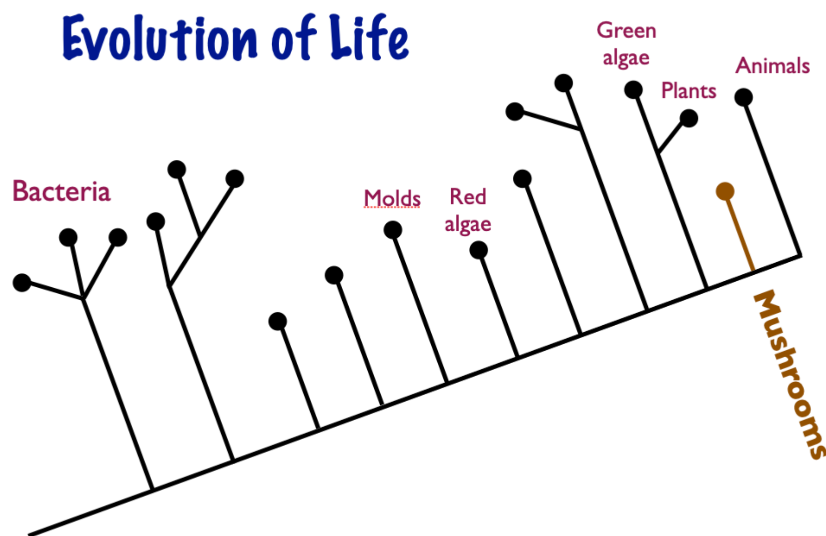


Figure 1. Evolution of life on Earth (Carroll, 2001). Reproduced with permission.

**1.2 Mushroom cultivation**

Mushrooms have been consumed for millennia, and farmed in Europe for the past three centuries, although it has only been in the last 90 years in Australia that there have been attempts to commercially produce mushrooms. Caves, quarries and disused railway tunnels have a constant temperature and humidity, making them ideally suited to growing mushrooms throughout most of the year. Railway tunnels under Sydney were used to cultivate mushrooms from 1933 during the cooler months using techniques common in 19$^{th}$ century Europe. It was not until the 1960s that modern methods of cultivation were employed to grow mushrooms year round in Australia (Miller, 2004).

Today, commercial button mushrooms (*Agaricus bisporus*) are grown in large specialised growing rooms with tightly regulated temperature, humidity, irrigation and compost ingredients (known as substrate) in which the mushrooms grow. The substrate is usually composed of wheat straw, poultry manure and gypsum, although there are many other possible ingredients. To this is added mushroom spores. From the spores grow the mycelium, a fine network of nutrient absorbing filaments that can be seen both above and within the growing substrate. In the wild, to colonise other areas, the mycelia send up fruiting bodies with gills full of reproductive spores. It is this fruiting body, which we consume as a mushroom, that is cut from the mycelium during harvesting. Technically, the organism classified as a mushroom, the mycelia, remains in the substrate!

As the fruiting body matures, the cap opens out, the ‘skirt’ covering the gills retracts, and the gills are now able to release their spores. The fruiting bodies are harvested at different sizes that includes small and medium sized button or cup mushrooms, and the larger field or flat mushroom. In the growing rooms, mushrooms tend to double in size about every 24 hours. At 5 cm diameter, the mushrooms in this study were not mature as there was still a ‘skirt’ covering the gills. This is the fresh mushroom size most popular with the Australian consumer. According to the Australian Mushroom Growers Association, button mushrooms are the $6^{th}$ most valuable horticultural crop in Australia, and 9 in 10 adults are mushroom consumers and 55% of households buy mushrooms at least weekly (https://australianmushrooms.com.au/did-you-know/).

**1.3 Early mushroom and vitamin D research**

From the early 1930s researchers observed that UV-irradiated ergosterol, abundant in mushrooms, would produce an antirachitic compound, one that would prevent rickets (Askew, Bourdillon, Bruce, & Jenkins, 1930; Pappenheimer, 1930; Pruess, Peterson, Steenbock, & Fred, 1931). Irradiated ergosterol was originally known as viosterol, before being termed vitamin $D_1$. Later, the structure of vitamin $D_1$ was found to be an adduct of two sterols, the stereoisomers tachysterol and ergocalciferol. The latter became the first isolated form of vitamin D and was labelled vitamin $D_2$; the term vitamin $D_1$ was no longer used in vitamin D terminology (DeLuca, 2011). Later, the version of vitamin D most commonly found in the animal kingdom, cholecalciferol, was termed vitamin $D_3$. Vitamin $D_2$ is primarily found in mushrooms, although it is also present in yeasts, algae, lichens and in small concentrations in some plants (L. J. Black, Lucas, Sherriff, Bjorn, & Bornman, 2017; Göring, 2018; Kessi-Pérez, González, Palacios, & Martínez, 2022).

Before there was a mushroom industry, people would collect seasonal mushrooms from the wild. It was a 1994 Finnish study of local wild mushrooms that stimulated the recent research on the vitamin D generation in edible mushrooms. The study found that five different wild mushroom species contained 3-30 $\mu$g $D_2$/ 100 g fresh weight generated by solar radiation (P. H. Mattila, Piironen, Uusi-Rauva, & Koivistoinen, 1994). Since then, many researchers have deliberately exposed fresh edible mushrooms to UV lamps and solar radiation (sunlight), usually generating more than the daily requirements of vitamin D in a 100 g serving of fresh mushrooms (Cardwell, Bornman, James, & Black, 2018).

If consumers wanted to increase the shelf life of their wild mushrooms, they would dry them outside in the sun or possibly in a low temperature oven. Sun-drying would further increase the vitamin $D_2$ concentration of wild mushrooms, and more recently, in commercial mushrooms (P.H. Mattila, Lampi, Ronkainen, Toivo, & Piironen, 2002; Rangel-Castro, Staffas, & Danell, 2002; Teichmann, Dutta, Staffas, & Jägerstad, 2007). With easy access to cultivated dried mushrooms available from supermarkets and specialty shops all year-round people are less inclined to go foraging for wild mushrooms.

### 1.4. Vitamin D

Vitamin D has been a key molecule in the evolution of life. Since at least 500 million years ago there has been photosynthesis of vitamin D in the sea, making it available to marine life. For example, phytoplankton produce vitamin $D_2$ from its precursor pro-vitamin $D_2$ (ergosterol), and the small crustacean krill have pro-vitamin $D_3$ (7-dehydrocholesterol), the precursor to vitamin $D_3$ (Holick, 2009). About 400 million years ago, some vertebrates emerged from the calcium-rich oceans to populate the land. They had to create a way to absorb calcium from their diet for a developing skeletal system needed to store calcium and support muscles in a gravity environment. With a change of habitat from the sea to land, during the evolution of animals, photosynthesis of vitamin D gradually became the role of the skin (Hernigou, Auregan, & Dubory, 2018).

In humans, skin exposure to a source of UV radiation, such as sunlight, will stimulate the conversion of pro-vitamin $D_3$ to pre-vitamin $D_3$, which in turn becomes vitamin $D_3$ through the action of body heat (Keegan, Lu, Bogusz, Williams, & Holick, 2013). At this stage vitamin $D_3$ has no specific metabolic function until it attaches to a vitamin D-binding protein and is then distributed to the liver to be hydroxylated to 25-hydroxyvitamin $D_3$ ($25(OH)D_3$), with a further hydroxyl group added by the kidney mitochondria to produce 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$). It is this latter molecule that interacts with vitamin D receptors in the gut to enhance the absorption of calcium for bone formation via osteoblasts (Holick, 2009). It is well established that vitamin D prevents rickets and osteomalacia. It has also been implicated in reducing the risk of cardiovascular disease, obesity, diabetes and hypertension, although the role if vitamin D in these conditions is not as clear (Bikle, 2014; Park, Pichiah, & Cha, 2018)

The production of vitamin $D_2$ is similar to vitamin $D_3$. The pro-vitamin $D_2$ (ergosterol) in phytoplankton, zooplankton, yeast and mushrooms absorbs the potentially dangerous UV

radiation, and in doing so, is converted to pre-vitamin $D_2$ and then thermally isomerised to vitamin $D_2$ (Keegan et al., 2013). Ergosterol is acting as a natural sunscreen to protect UV radiation sensitive molecules, such as DNA and RNA, from damage (Holick, 2003). In mushrooms, the process begins with the conversion of ergosterol to pre-vitamin $D_2$ by the action of UV radiation, mainly UV-B (280-315 nm), splitting the 9,10 bond of the B ring, with a maximal absorption at 281 nm (Schümmer, Stangl, & Wätjen, 2021) (Fig 2). The evolutionary selection of ergosterol in yeast and mushrooms, in preference to other sterols, seems to be due to its additional protective role against mechanical and oxidative stress caused by the transition between wet and dry conditions experienced through their lifecycle (Dupont et al., 2012). Like vitamin $D_3$, vitamin $D_2$ must also undergo two hydroxylations before it is metabolically active in animals as 1,25-dihydroxyvitamin $D_2$ (1,25$(OH)_2D_2$).

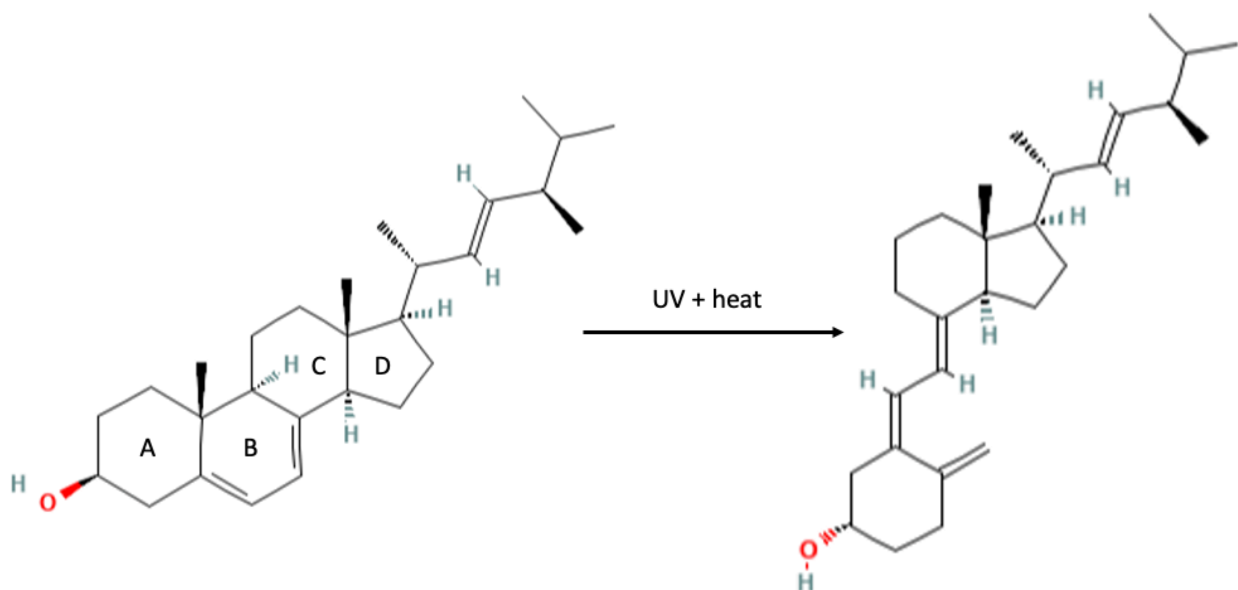


Fig 2. Ergosterol with B ring intact (L), forming ergocalciferol (R) after exposure to UV radiation and heat.

Although this thesis is focussed on vitamin $D_2$, and to a lesser extent on 25-hydroxyvitamin $D_2$, UV-irradiated mushrooms also contain vitamins $D_3$ and $D_4$ (Keegan et al., 2013; Schümmer et al., 2021). The precursor for vitamin $D_4$ (22,23-dihydroergosterol) is present in edible mushrooms, and with the exposure to UV radiation vitamin $D_4$ (22-dihydroergocalciferol) is formed, which has been identified in all commonly consumed mushrooms, such as enoki, shiitake, oyster, chanterelle, maitake and the common button mushroom (Keegan et al., 2013; K. M. Phillips, Horst, Koszewski, & Simon, 2012). The function of vitamin $D_4$ in human health is not clear.

## 1.5 Vitamin D recommended dietary intake

Different regions of the world have different recommendations for vitamin D intake. The recommended intake of vitamin D is 5-15 µg/day in Australia and New Zealand, depending on age (National Health and Medical Research Council, 2006), 15-20 µg/day (600-800 IU) in the USA (Institute of Medicine, 2011), 15 µg/day as set by the European Food Safety Authority (EFSA, 2017), 15-20 µg/day for Canadians (Government of Canada, 2010), 10 µg/day in the United Kingdom (Scientific Advisory Committee on Nutrition, 2016), and 10-20 µg/day in the Nordic countries (Nordic Council of Ministers, 2014) (Table 1).

Table 1. Recommended daily intake of vitamin D (µg/day) according to age across different regions of the world.

| Region | Age (years) | | | | |
|---|---|---|---|---|---|
| | **1-18** | **19-30** | **31-50** | **51-70** | **71+** |
| United States of America [a] | 15 | 15 | 15 | 15 | 20 |
| Canada [b] | 15 | 15 | 15 | 15 | 20 |
| United Kingdom [c] | 10 | 10 | 10 | 10 | 10 |
| Europe [d] | 15 | 15 | 15 | 15 | 15 |
| Australia and New Zealand [e] | 5 | 5 | 5 | 10 | 15 |
| Nordic countries [f] | 10 | 10 | 10 | 10 | 20 |

[a] USA, Recommended Dietary Allowance (RDA) (Institute of Medicine, 2011); [b] Canada, Adequate Intake (AI) (Government of Canada, 2010); [c] UK, Reference Nutrient Intake (RNI) (Scientific Advisory Committee on Nutrition, 2016); [d] Europe, AI (EFSA, 2017); [e] Australia and NZ, AI (National Health and Medical Research Council, 2006); [f] Nordic Nutrition Recommendations 2012 (Nordic Council of Ministers 2014)

Consumption of vitamin D in Australia was initially reported to be 2–3 µg/day (Nowson & Margerison, 2002; Shrapnel & Truswell, 2006), then later thought to be at least 4.3 µg/day when including 25(OH)$D_3$ in the calculations (J. Liu et al., 2015). Those estimated intakes of vitamin D have been challenged as the Australian Food Composition Database is updated. More recently, using more accurate vitamin D composition data, the intake has been estimated to be 1.84-3.25 µg/day (Dunlop et al., 2022), which is still less than the recommended daily intake in Australia, and other countries and regions (Table 1). The main sources of vitamin D in Australia are fish, egg yolk and vitamin D-fortified foods such as table margarine, some milks and breakfast cereals, with red meats and chicken providing lesser amounts. Frequently consumed food sources of vitamin D are shown in Table 2.

Table 2. Common Australian dietary sources of vitamins $D_3$ and $D_2$ per 100 g in Australia.

| Food source | Vitamin $D_3$ µg/100g | Vitamin $D_2$ µg/100g |
|---|---|---|
| Salmon, canned | 19.25 | ND |
| Salmon, Atlantic | 4.99 | ND |
| Sardines, baked in foil | 4.90 | Trace |
| Fish, Barramundi | 3.90 | ND |
| Breakfast cereals, ready to eat, vitamin D fortified | 11.90 | 0.13 |
| Table margarine | 8.45 | 3.64 |
| Egg, boiled | 1.44 | ND |
| Milk, vitamin D fortified | 0.45 | ND |
| Chicken, breast, fried no oil | 0.15 | ND |
| Egg, boiled | 1.44 | ND |
| Beef, mince, regular fat, fried no oil | 0.21 | 0.22 |
| Lamb, chops, grilled | Trace | 0.24 |

Source: Australian Food Composition Database (https://www.foodstandards.gov.au/science/monitoringnutrients/afcd/Pages/Data-provided-by-food-companies-and-organisations.aspx)
ND, not detected

Other countries have also shown a lower dietary intake than government recommendations, commonly less than 5 µg/day (Herrick et al., 2019; Keily & Black, 2012). The discrepancy between actual and recommended vitamin D intake indicates that dietary sources alone are unlikely to lead to adequate vitamin D status. There is current interest in fortifying common foods, such as milk and bread, to boost the dietary intake of consumers. A potential additional source of D vitamers are UV-exposed fresh and dried mushrooms, especially to vegans and vegetarians who will avoid the more abundant animal sources of vitamin D.

### 1.6 Common button mushrooms (*Agaricus bisporus*)

The mushrooms studied in this project are *Agaricus bisporus*, which comprise more than 90% of the mushrooms purchased in Australia. They are one of the four most commonly consumed mushrooms in the world, along with *Lentinus edodes* (shiitake), *Pleurotus ostreatus* (oyster), and *Auricularia auricula* (wood ear) (Royse, Baars, & Tan, 2017).

The gills on the underside of the mushroom generate more vitamin $D_2$ when exposed to UV radiation as they have a higher concentration of ergosterol compared to other parts of the mushroom (Fig. 3) (Krings & Berger, 2014; Perera, Jasinghe, Ng, & Mujumdar, 2003). The cap has the next highest concentration of ergosterol, with the stipe or stalk having the least.

Although the gills were skirted in all the mushrooms selected for the studies in this thesis, the underside was always the side exposed to the pulsed UV-radiation so that all the results were comparable. Had the mushrooms been more mature, the skirt retracted, and the gills exposed directly to the UV radiation, they would have produced more D vitamers at the same radiation dose. In a commercial mushroom farm producing vitamin D mushrooms it is unlikely that the mushrooms will be evenly orientated when exposed to a UV lamp. If mushrooms are pre-packed (e.g. 500 g packs) prior to UV-irradiation it is likely that there will be two layers of mushrooms, with the top layer shielding the lower layer(s) from exposure to UV radiation (Koyyalamudi, Jeong, Pang, Teal, & Biggs, 2011).

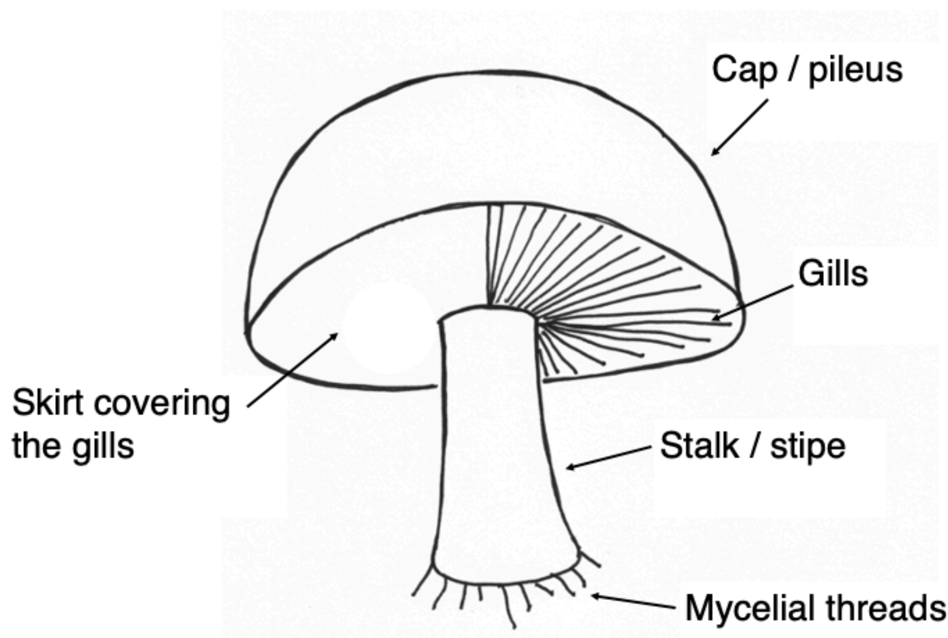


Figure 3. Basic morphology of the mushroom *Agaricus bisporus*. The gills are revealed as the mushroom matures. (Illustration by G. Cardwell)

Commercial button mushrooms grow well without light. The only time they are likely to be exposed to light is when they are being harvested (always by hand in Australia) under fluorescent lamps. Fluorescent light has a visible light wavelength of 400-700 nm, a spectrum that does not include the UV bandwidth, and therefore does not trigger the vitamin D-generating process in mushrooms.

Published papers commonly report 90-92% moisture in fresh mushrooms purchased from retail outlets where 24-72 h may have passed since they were harvested. Mushrooms begin to lose water from the moment they are harvested via transpiration, with the rate dependent on the ambient temperature and humidity (Mahajan, Oliveira, & Macedo, 2008). If fresh mushrooms are weighed on a scale accurate to 0.001 g, weight loss (transpiration) becomes evident second by second. For this reason, all the fresh samples in the studies of this thesis were measured to 0.1 g as a higher level of accuracy is impracticable and misleading. To minimise weight loss due to post-harvest transpiration, commercial mushrooms are stored at 5 ºC and transported in refrigerated trucks. The mushrooms are packed in 4 kg boxes; however, they are packed overweight so that wholesalers and retailers still receive boxes weighing at least 4 kg. It is wise for the retailer to properly care for their mushrooms; a loss of, say, 5% moisture means a 5% drop in purchase price (and profit) to the retailer. In addition, as moisture is lost the mushrooms begin to shrivel and become less attractive to the customer.

## 1.7 Summary

There are many people in the world without access to refrigeration, or who prefer the convenience of dried foods, or choose non-animal sources of nutrition, therefore a better understanding of the D-vitamer content in dried mushrooms is needed. There is a global future for vitamin D-enhanced dried mushrooms both as a food and, if the dried mushrooms are ground to a powder, as a supplement or functional food ingredient. It is important to know the ideal sequence of drying and irradiating mushrooms, the rate of decline in the concentration of D vitamers during storage, and the retention during cooking if UV-irradiated dried mushrooms are promoted as a D vitamer source to the public.

**Aims and objectives**

As there are very few studies on the generation of D vitamers in UV-irradiated whole dried mushrooms as stored and consumed by the public, the overall aim of this thesis was to determine if vitamin D-enriched dried mushrooms have commercial viability for both producer and consumer.

***Objective 1***

The first objective was to conduct a review of the literature regarding UV-irradiated mushrooms being a potential dietary source of D vitamers, and from that to identify gaps in the research, especially the research on UV-irradiated dried mushrooms and D vitamer generation (Chapter 2). Such a comprehensive review had not been published before.

The following gaps in our knowledge were identified from the review:

a) There were only four studies that used pulsed UV irradiation to generate vitamin $D_2$, and all four were conducted on fresh mushrooms. There were no studies of pulsed UV radiation in dried mushrooms.

b) There were no studies examining the effect of the sequence of UV-irradiation and drying of mushrooms on the generation of D vitamers.

c) There was no study monitoring the retention of vitamin $D_2$ in dried mushrooms during storage after pulsed UV radiation; there was a single study in button mushrooms which were exposed to a UV-B lamp for 30 minutes.

d) There were no studies on the D vitamer retention in irradiated dried mushrooms that had been cooked.

This thesis addresses those gaps with the following three objectives:

***Objective 2***

To verify the method of analysing vitamin $D_2$ and 25-hydroxyvitamin $D_2$ (25(OH)$D_2$) concentrations within a mushroom matrix, and then determine the ideal sequence of exposing mushrooms to UV radiation and air-drying for generating D vitamers.

***Objective 3***

The next objective was to analyse the retention of D vitamers in pulse UV-irradiated, dried mushrooms when stored for up to 12 months in conditions normally experienced in an Australian household pantry.

***Objective 4***

The final objective was to determine the retention of D vitamers in UV-irradiated, dried mushrooms after being rehydrated and then cooked by three different common methods.

Chapters 3-5 include the studies that fulfilled the last three objectives.

**Chapter 2: A review of mushrooms as a potential source of dietary vitamin D**

**Objective 1:** Conduct a review of the literature regarding mushrooms being a potential dietary source of D vitamers, and identify gaps in the research, especially on UV-irradiated dried mushrooms and D vitamer generation.

Below is the final submitted version of the manuscript that was published by the open access journal *Nutrients* in October 2018. Following the review, relevant studies that have been published since October 2018 have been outlined.

The published literature review (Nutrients 2018, 10, 1498; doi:10.3390/nu10101498) is available here: https://www.mdpi.com/2072-6643/10/10/1498.

## 2.1. A review of mushrooms as a potential source of dietary vitamin D

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**Abstract:** When commonly consumed mushroom species are exposed to a source of ultraviolet (UV) radiation, such as sunlight or a UV lamp, they can generate nutritionally relevant amounts of vitamin D. The most common form of vitamin D in mushrooms is $D_2$, with lesser amounts of vitamins $D_3$ and $D_4$, while vitamin $D_3$ is the most common form in animal foods. Although the levels of vitamin $D_2$ in UV-exposed mushrooms may decrease with storage and cooking, if they are consumed before the 'best before' date, vitamin $D_2$ is likely to remain above 10 μg/100 g fresh weight, which is more than most vitamin D-containing foods and similar to the daily requirement of vitamin D recommended internationally. World-wide mushroom consumption has increased markedly in the past four decades, and mushrooms have the potential to be the only non-animal, unfortified food source of vitamin D that can provide a substantial amount of vitamin $D_2$ in a single serve. This review examines the current information on the role of UV radiation in enhancing the concentration of vitamin $D_2$ in mushrooms, and the effects of storage and cooking on vitamin $D_2$ content, as well as the bioavailability of vitamin $D_2$ from mushrooms.

**Keywords:** Vitamin D; Mushroom; UV radiation; Button mushroom; *Agaricus bisporus*; Shiitake mushroom; *Lentinula edodes;* Oyster mushroom; *Pleurotus ostreatus*

## 1. Introduction

Vitamin D stimulates the synthesis of the calcium-transport protein in the small intestine, enhancing the absorption of dietary calcium, and thereby reducing the risk of osteomalacia in adults and rickets in children (Jones, 2014; Lips, 2006). Adequate vitamin D is also important for muscle function and reducing the risk of falls in the elderly (Girgis, Clifton-Bligh, Hamrick, Holick, & Gunton, 2013), and may help protect against some cancers, respiratory disease in children, cardiovascular disease, neurodegenerative diseases, and both type 1 and type 2 diabetes (Ford et al., 2014; Gaksch et al., 2017; Hossein-nezhad & Holick, 2013; Koduah, Paul, & Dörr, 2017), although current evidence for non-skeletal benefits is inconclusive (Theodoratou, Tzoulaki, Zgaga, & Ioannidis, 2014). Although vitamin D is classified as a vitamin, it can be produced by the body in sufficient quantities when the skin is exposed to ultraviolet (UV) radiation from the sun (Jones, 2014). If sunlight exposure is limited, dietary sources of vitamin D are required to maintain healthy circulating 25-hydroxyvitamin D (25(OH)D) concentrations. It is estimated that 1 billion people worldwide are vitamin D deficient (25(OH)D concentrations $\leq$ 50 nmol/L), with prevalence in excess of 50% being commonly reported in population-based studies.

The two main dietary forms of vitamin D are vitamin $D_2$, found in fungi and yeast, and $D_3$, found in animals; lesser amounts of vitamin $D_3$ and $D_4$ are also found in fungi (Keegan et al., 2013; K. M. Phillips et al., 2012; Taofiq, Fernandes, Barros, Barreiro, & Ferreira, 2017; Urbain, Valverde, & Jakobsen, 2016) (Figure1). Few foods in the Western diet are a good source of vitamin D, with the best naturally occurring dietary source being oily fish. Some countries have liberal fortification policies, with foods such as milk, margarine, breakfast cereals and juices fortified with vitamin D (Calvo, Whiting, & Barton, 2005; Lamberg-Allardt, 2006). Sun-dried and UV radiation-exposed mushrooms are a potentially important source of dietary vitamin D (as vitamin $D_2$) (Mau, Chen, & Yang, 1998; Nölle, Argyropoulos, Ambacher, Muller, & Biesalski, 2016; Simon, Phillips, Horst, & Munro, 2011). Vitamin D-enhanced mushrooms are the only non-animal food product with substantial amounts of bioavailable vitamin D and, as such, have the potential to be a primary source of dietary vitamin D for vegans and vegetarians.

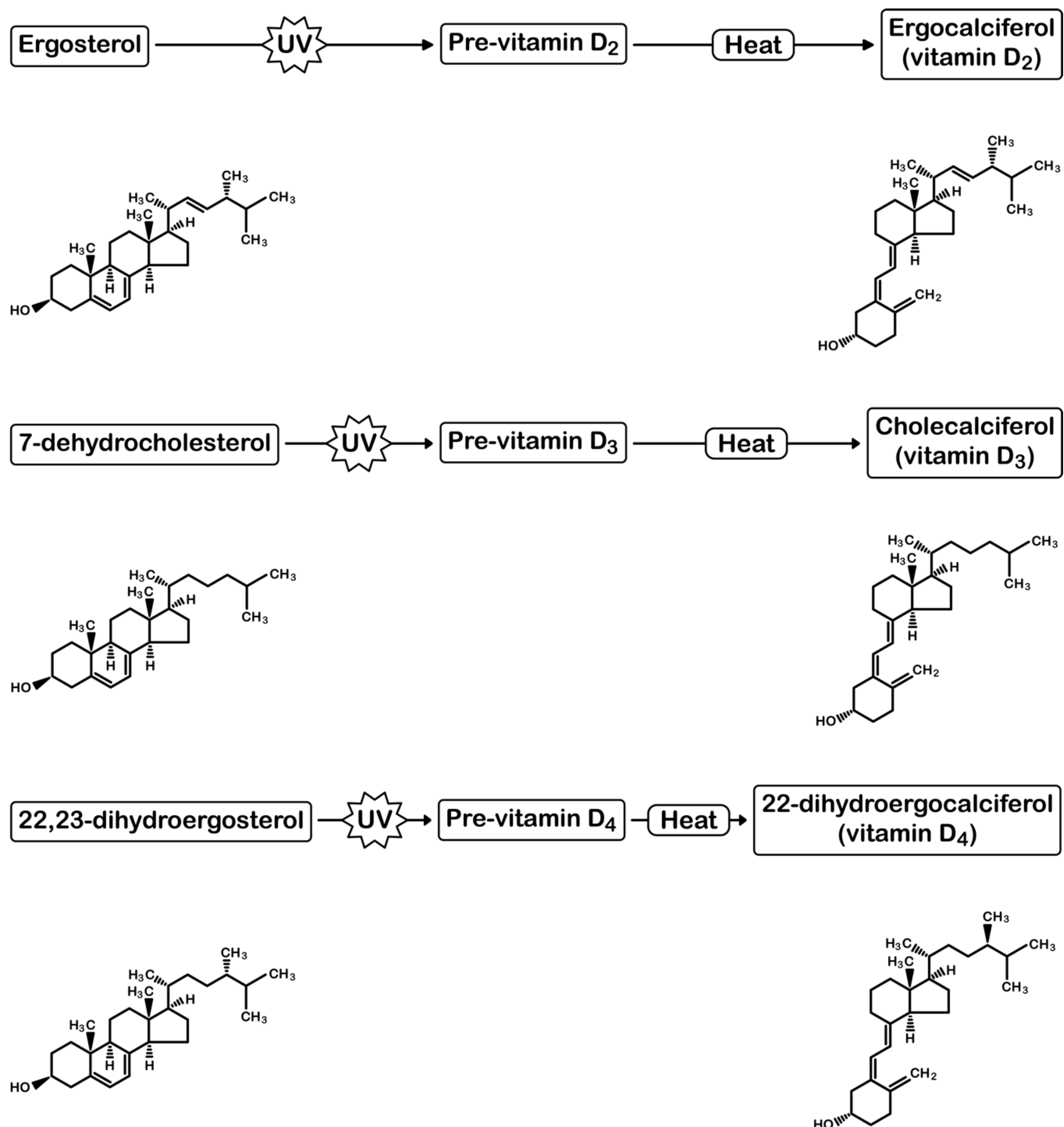


**Figure 1.** Structures of vitamin $D_2$, $D_3$ and $D_4$ and their precursors.
**U**V, ultraviolet radiation.

This review addresses the potential for a good dietary source of vitamin D in mushrooms. We considered mushrooms exposed to different sources of UV radiation (solar radiation, UV fluorescent lamp and pulsed UV lamp) to gauge the potential for increasing vitamin $D_2$ content, and whether the amount of vitamin $D_2$ generated was nutritionally significant. We focussed on the three most commonly consumed mushrooms worldwide: the button mushroom *Agaricus bisporus* (30% of worldwide consumption), oyster mushrooms *Pleurotus* (all species: 27% of worldwide consumption) and shiitake mushrooms *Lentinula edodes* (17% of worldwide consumption), together comprising approximately three quarters of all mushrooms consumed (Royse, 2014). Studies of other edible mushroom species were included where context was needed or if there was very little information on the most popular mushrooms. This review is

limited to English-language publications and, for consistency and comparability, includes only those studies where vitamin D in mushrooms was measured using high performance liquid chromatography.

## 2. Requirements and intake of dietary vitamin D

The recommended intake of vitamin D is 5-15 µg/day (200-600 IU) in Australia and New Zealand, depending on age (National Health and Medical Research Council, 2006), 15-20 µg/day (600-800 IU) in the USA (Institute of Medicine, 2011), 15 µg/day (600 IU) as set by the European Food Safety Authority (EFSA, 2017), 15-20 µg/day (600-800 IU) for Canadians (Government of Canada, 2010), and 10 µg/day (400 IU) in the United Kingdom (Scientific Advisory Committee on Nutrition, 2016) (Table 1).

**Table 1.** Examples of recommended daily intakes of vitamin D (µg/day) across different regions.

| | **Age (years)** | | | | |
|---|---|---|---|---|---|
| | **1-18** | **19-30** | **31-50** | **51-70** | **71+** |
| United States of America [a] | 15 | 15 | 15 | 15 | 20 |
| Canada [b] | 15 | 15 | 15 | 15 | 20 |
| United Kingdom [c] | 10 | 10 | 10 | 10 | 10 |
| Europe [d] | 15 | 15 | 15 | 15 | 15 |
| Australia and New Zealand [e] | 5 | 5 | 5 | 10 | 15 |

[a] USA, Recommended Dietary Allowance (RDA) (Institute of Medicine, 2011); [b] Canada, Adequate Intake (AI) (Government of Canada, 2010); [c] UK, Reference Nutrient Intake (RNI) (Scientific Advisory Committee on Nutrition, 2016); [d] Europe, AI (EFSA, 2017); [e] Australia and NZ, AI (National Health and Medical Research Council, 2006).

Estimates of the dietary intake of vitamin D in the USA are 5-6 µg/day in adult males and 3.5-4.5 µg/day in adult females, although the intake of those taking vitamin D supplements may reach the Adequate Intake (AI) (Bailey, 2010). Canadian adults obtain an average of 5.8 µg/day from food, which includes vitamin D-fortified foods such as milk (Vatanparast, 2010). European intake of vitamin D is estimated to be 2-4 µg/day (Calvo et al., 2005). In the Irish population, the median intake of total vitamin D in adults is estimated as 3.5 µg/day, and 3.7 µg/day in those consuming vitamin D-fortified foods (L.J. Black, Walton, Flynn, Cashman, & Kiely, 2015). All these estimates are higher than the estimated Australian adult dietary intake of 2-3 µg/day (Shrapnel & Truswell, 2006), where vitamin D fortification is more restricted. However, with improved analytical methods for vitamin D and its metabolites in food, the previously reported estimates of vitamin D intake in Australia have been disputed and may be as high as 4.3 µg/day from animal food alone when including both vitamin $D_3$ and its hydroxylated metabolite, 25-hydroxyvitamin $D_3$ (25(OH)$D_3$) (J. Liu et al., 2015).

The discrepancy between actual and recommended vitamin D intakes indicates that dietary sources alone are unlikely to lead to adequate vitamin D status.

## 3. Vitamin D metabolism in mushrooms

There are five biological kingdoms: Animalia, Plantae, Fungi, Protista (e.g. algae) and Monera (e.g. bacteria) (Margulis & Chapman, 2010). Mushrooms reside in the fungal kingdom, making them very different biological entities compared to plants and animals, despite being considered a vegetable from a culinary perspective. Unlike plants, mushrooms have high concentrations of ergosterol in their cell walls, playing a similar role as cholesterol plays in animals, i.e. strengthening cell membranes, modulating membrane fluidity and assisting intracellular transport (Weete, Abril, & Blackwell, 2010). The presence of both ergosterol and vitamin $D_2$ in mushrooms was first reported in the early 1930s (Quackenbush, Peterson, & Steenbock, 1935). When exposed to UV radiation, ergosterol in the mushroom cell wall is transformed to pre-vitamin $D_2$, which is then thermally isomerised in a temperature-dependent process to ergocalciferol, commonly known as vitamin $D_2$ (Jasinghe, Perera, & Sablini, 2007; Keegan et al., 2013). Through a similar process, pro-vitamin $D_4$ (22,23-dihydroergosterol) from mushrooms is converted to vitamin $D_4$ (K. M. Phillips et al., 2012). All commonly consumed mushrooms have provitamin $D_4$, making them a potential source of vitamin $D_4$ if exposed to UV radiation (K. M. Phillips et al., 2012). In general, there is a positive correlation between $D_2$ and $D_4$ in mushrooms (K. M. Phillips et al., 2012).

## 4. Vitamin D content of fresh mushrooms

### *4.1. Fresh wild mushrooms*

The recent interest in the vitamin $D_2$ content of mushrooms began with the discovery that wild edible Finnish mushrooms, the funnel chanterelle (*Cantharellus tubaeformis*), sampled in late summer and early autumn provided 3-30 µg $D_2$/100 g fresh weight (FW), compared with less than 1 µg $D_2$/100 g FW in the button mushroom purchased from retail outlets (P. H. Mattila et al., 1994). Since then, large amounts of vitamin $D_2$ have been found in wild funnel chanterelles (21.1 µg $D_2$/100 g FW), *Cantharellus cibarius* (10.7 µg $D_2$/100 g FW) and *Boletus edulis* (58.7 µg $D_2$/100 g FW) (Teichmann et al., 2007). A smaller amount of vitamin $D_2$ (1.5 µg/100 g FW) was reported in wild *Agaricus* species in Denmark (Kristensen, Rosenqvist, & Jakobsen, 2012).

### *4.2. Fresh retail mushrooms*

Most fresh retail mushrooms sold in the UK, Europe, North America, Australia and New Zealand, especially the button mushroom, are grown in atmospherically controlled growing rooms, then harvested and taken to market and retail outlets in refrigerated transport. As they are usually grown in darkness, the only time they are likely to be exposed to light is during picking under fluorescent lights, which usually emit little or no UV radiation. Hence, the vitamin $D_2$ content of retail fresh button mushrooms sold around the world is commonly reported to be less than 1 µg/100 g FW (Koyyalamudi, Jeong, Song, Cho, & Pang, 2009; P. H. Mattila et al., 1994; K. M. Phillips et al., 2012; K. M. Phillips et al., 2011; Simon et al., 2011; Teichmann et al., 2007). As 100 g is considered to be a realistic serve of mushrooms (approximately three button mushrooms), a typical serve will provide negligible vitamin $D_2$. The National Nutrient Database of the United States Department of Agriculture lists shiitake, white button and oyster mushrooms as all containing vitamin $D_2$ at less than 1 µg/100 g FW (United States Department of Agriculture, 2018).

*4.3. Fresh mushrooms exposed to sunlight*

When fresh button mushrooms are deliberately exposed to midday sunlight for 15-120 min they generate significant amounts of vitamin $D_2$, usually in excess of 10 µg/100 g FW (Kristensen et al., 2012; K.M. Phillips & Rasor, 2013; Simon et al., 2011; Urbain & Jakobsen, 2015), which is approaching the daily requirement of vitamin D recommended in many countries (Table 1). However, the amount of vitamin $D_2$ generated depends on the time of day, season, latitude, weather conditions, and exposure time. Since they have a higher surface area to volume (hence, more ergosterol is exposed), sun-exposed sliced mushrooms produce more vitamin $D_2$ than whole mushrooms from the same amount of UV radiation exposure (K.M. Phillips & Rasor, 2013; Urbain & Jakobsen, 2015; Urbain et al., 2016). At midday in mid-summer in Germany, the vitamin $D_2$ content of sliced mushrooms was as high as 17.5 µg/100 g FW after 15 min of sun exposure and 32.5 µg/100 g FW after 60 min of sun exposure (Urbain & Jakobsen, 2015). An unpublished Australian study on whole button mushrooms determined the vitamin $D_2$ content after exposure to the midday winter sun in July in Sydney (personal communication, J. Ekman, Applied Horticultural Research, 12 August 2013). Sun exposure to a single layer of small button mushrooms was sufficient to generate 10 µg $D_2$/100 g FW after 1 h, while large button mushrooms took 2 h to generate the same amount of vitamin $D_2$.

*4.4. Fresh mushrooms exposed to UV radiation from lamps*

An efficient way to produce nutritionally relevant amounts of vitamin $D_2$ is to expose mushrooms to specific, controlled levels of UV radiation via a fluorescent UV lamp or a pulsed UV lamp. Mushrooms will generate vitamin $D_2$ in response to exposure to UV radiation both during the growing phase and post-harvest; however, commercial growers use UV lamps post-harvest for practical reasons. Fresh mushrooms, when deliberately exposed to a UV radiation source post-harvest, will generate significant amounts of vitamin $D_2$, often reaching 40 µg/g dried mass (DM) (*ca* 320 µg/100 g FW) (Jasinghe & Perera, 2006; Kalaras, Beelman, & Elias, 2012; Ko, Lee, Lee, & Park, 2008; Koyyalamudi et al., 2011; Koyyalamudi et al., 2009; Mau et al., 1998; Simon et al., 2011; Urbain & Jakobsen, 2015; Urbain et al., 2016; Wittig, Krings, & Berger, 2013). The most effective wavelength to stimulate the production of vitamin $D_2$ in mushrooms is UV-B radiation (280-315 nm) (Jasinghe & Perera, 2006). Some researchers have used UV-A (315-400 nm) (Jasinghe & Perera, 2005, 2006; Jasinghe, Perera, & Barlow, 2006; Teichmann et al., 2007) and UV-C (<280 nm) radiation (Guan et al., 2016; Huang, Cai, & Xu, 2016; Jasinghe & Perera, 2005, 2006; Koyyalamudi et al., 2009; Mau et al., 1998; Teichmann et al., 2007), however UV-A radiation was not effective at increasing vitamin $D_2$ concentrations in all cases (Teichmann et al., 2007).

In fresh shiitake mushrooms, ergosterol concentrations are highest in the gills, followed by the cap and stalk, with the gills having twice the concentration of ergosterol as the cap (Jasinghe & Perera, 2005; Perera et al., 2003). Subsequently, the gills of the shiitake mushroom generate more vitamin $D_2$ when exposed to UV-B radiation than the cap or stalk (Ko et al., 2008), with the gills generating up to four times the vitamin $D_2$ than the cap (22.8 µg/g DM *vs* 5.2 µg/g DM) (Jasinghe & Perera, 2005). Whole oyster mushrooms have been shown to generate more than twice the vitamin $D_2$ than shiitake at the same UV-exposure level (Jasinghe & Perera, 2005, 2006): when sliced and exposed to 60 min of UV-B lamp radiation, oyster mushrooms produced up to 140 µg/g DM (Jasinghe & Perera, 2005, 2006; Krings & Berger, 2014; Wittig et al., 2013; Wu & Ahn, 2014). Irradiation intensity was the most critical factor in determining vitamin $D_2$ concentration: 90 min of exposure to UV-B radiation at 1.14 $W/m^2$ at 28ºC were the optimal conditions for generating vitamin $D_2$, producing 240 µg/g DM (Wu & Ahn, 2014). UV-B lamp irradiation has also been shown to increase the vitamin $D_4$ concentration in oyster mushrooms from 0 to 20 µg/g DM at 20ºC after only 30 min (Krings & Berger, 2014).
The influence of temperature on vitamin $D_2$ production has not been investigated in detail, although two studies suggest that temperatures between 25-35ºC may be ideal for commercial purposes. One study showed that vitamin $D_2$ production in whole oyster mushrooms increased

from 152 µg/g DM to 178 µg/g DM as the temperature increased from 15ºC to 35ºC (Wu & Ahn, 2014), while results from another study showed that the optimum conversion of ergosterol to vitamin $D_2$ in shiitake mushrooms occurred at 35ºC and 78% moisture, producing *ca* 50 µg/g DW (Jasinghe & Perera, 2005).

As demonstrated in two studies, nutritionally relevant concentrations of vitamin $D_2$ (10 µg/100 g FW) in whole mushrooms can also be effectively achieved with a commercial pulsed UV lamp within a very short time-period of 1-2 s (3-6 pulses) (Kalaras et al., 2012; Koyyalamudi et al., 2011). In contrast, it can take several minutes to generate the same concentration of vitamin $D_2$ using a UV fluorescent lamp. Therefore, pulsed UV radiation may be the most efficient method of increasing vitamin $D_2$ concentrations in mushrooms. In button mushrooms, three pulses (1 s) of UV radiation generated 11.9 µg $D_2$/g DM (Kalaras et al., 2012), and nine pulses (3 s) generated 20 µg $D_2$/g DM (Koyyalamudi et al., 2011). The maximum concentration of vitamin $D_2$ (27 µg/g DM) was reached after 12 pulses (4 s) (Kalaras et al., 2012). The laboratories in both studies used similar pulsed UV lamp systems (Xenon Corporation), which produce a pulse of high energy UV radiation (505J/pulse) that is able to generate vitamin $D_2$ deep within the 'flesh' of the mushroom. The mushrooms received either 1.1 J/cm$^2$ per pulse (Koyyalamudi et al., 2011) or 0.791 J/cm$^2$ per pulse (Kalaras et al., 2012). The concentration of vitamin $D_2$ generated depends on the type and orientation of the mushroom, whether they are sliced or whole, distance from the lamp housing, size of the mushroom and the total number of pulses received.

## 5. Dried mushrooms exposed to UV radiation from lamps

Commercial dried mushrooms have a much longer shelf life than fresh mushrooms, often with a 'best before' date 2-3 years after packaging. They have about 15% of the original weight of fresh mushrooms, making them cheaper to transport and, potentially, a cheaper source of vitamin $D_2$.

### *5.1. Sun-dried mushrooms*

Sun-drying is one method used for drying mushrooms in Asian countries. Analysis of the vitamin $D_2$ and ergosterol content of 35 species of dried mushrooms sold in China revealed they contained significant amounts of vitamin $D_2$, with an average of 16.9 µg/g DM (range of 7-25 µg/g DM (Huang et al., 2016). No details were provided on the method of drying, nor the time

since the initial drying. The moisture content of the commercial dried mushrooms varied, although the majority contained 3-7% moisture.

*5.2. Hot-air dried mushrooms*

Mushroom collectors often pick mushrooms in the wild, dry them using a hot-air method, then store the dried mushrooms for months or years. Mimicking this process showed that chanterelles (*Cantharellus cibarius*) collected from Swedish forests had vitamin $D_2$ between 0.12-6.3 µg/g DM after being hot-air dried and stored in darkness for 2-6 years (Rangel-Castro et al., 2002). In a study of button, shiitake and oyster mushrooms, the authors suggested that 60°C is the optimum air-drying temperature post UV-B exposure, since obvious discolouration occurred above 60°C (Nölle, Argyropoulos, Müller, & Biesalski, 2018). When shiitake mushrooms were dried under laboratory conditions, the conversion of ergosterol to vitamin $D_2$ was most efficient when the mushroom contained 70% moisture and received UV-B radiation (ranging 290-320 nm in this study) for 2 h, producing 25 µg $D_2$/g DM (*ca* 200 µg $D_2$/100 g FW) (Perera et al., 2003). As the mushrooms dried in a desiccator over seven days, their ability to generate vitamin $D_2$ decreased as the moisture content dropped from 70% to 30%, although the vitamin $D_2$ concentrations were still nutritionally significant at 30% moisture content (15 µg $D_2$/g DM; *ca* 120 µg $D_2$/100 g FW).

*5.3. Freeze-dried mushrooms*

Mushrooms that have been freeze-dried will have close to zero moisture, resulting in 8-10% of the weight of the original mushroom (unlike dried mushrooms which still have a small water content, possibly 5%). Freeze-dried button, shiitake and oyster mushrooms generated more vitamin $D_2$ after exposure to UV-B radiation than did hot-air dried mushrooms (Sławińska et al., 2016). The authors suggested that the internal pore structures of the freeze-dried mushrooms facilitated the penetration of UV-B radiation. From having no detectable vitamin $D_2$ content, freeze-dried oyster mushrooms generated 34.6 µg $D_2$/g DM, shiitake 60 µg $D_2$/g DM, and button mushrooms 119 µg $D_2$/g DM after 30 min of exposure to radiation. Hot air-dried mushrooms with a moisture content of 6-8.3% produced 32-81 µg $D_2$/g DM over the same time frame.

Different variables (time of exposure, temperature, and exposure to UV-B radiation) can influence vitamin $D_2$ production in button mushrooms that are freeze-dried then powdered. For example, the ideal conditions for generating vitamin $D_2$ from button mushroom powder was

using a UV-B lamp (range 280-360 nm) with an irradiance of 1.36 $W/m^2$ for 10 min and at a temperature of 26ºC, producing 740 µg $D_2$/g powder (N. K. Lee & Aan, 2016). When freeze-dried, powdered shiitake mushrooms were exposed to 20 pulses from a Xenon RC 801 pulsed light system (UV range 190-700 nm) the concentration of vitamin $D_2$ generated was 37 µg/g DM, while 60 pulses generated 62 µg/g DM (Chien, Yang, Lin, & Mau, 2017).

## 6. Stability of vitamin $D_2$ in vitamin D-enhanced mushrooms after storage and cooking

### *6.1. Storage*

Analysis of the retention of vitamin $D_2$ in both fresh and dried mushrooms exposed to UV radiation has mainly been done after refrigeration at 2-4ºC. Fresh button mushrooms stored at 2.2ºC showed a first order kinetics decline in vitamin $D_2$ concentration, with a predicted decline to a concentration of 1.75 µg/g DM at 14 days (Roberts, Teichert, & McHugh, 2008). The vitamin $D_2$ concentration in sliced button mushrooms dropped from 12 µg/g DM to 8-9 µg/g DM after 3-11 days storage at 3ºC (Kalaras et al., 2012). Oyster and shiitake mushrooms stored at 4ºC, showed a slight increase in vitamin $D_2$ concentrations in the first 24 h of storage before they gradually reduced over 10 days to about one third to a half of their highest post UV-exposure level (Sławińska, Fornal, Radzki, Jablonska-Rys, & Parfieniuk, 2017). In one study, the vitamin $D_2$ concentration in button mushrooms gradually increased from 3.5 µg/g DM to 8.1 µg/g DM during storage at 4ºC for 6 days, before dropping on day 7 and 8, while for oyster and shiitake their vitamin $D_2$ concentration dropped gradually over 10 days (Sławińska et al., 2017). However, other studies did not show substantial vitamin $D_2$ losses when mushrooms were refrigerated. There was virtually no degradation of vitamin $D_2$ when button mushrooms were refrigerated at 4ºC for eight days (Koyyalamudi et al., 2009), or for 7 and 14 days (Guan et al., 2016). Similar concentrations of vitamin $D_2$ were found at 1 and 4 days in button mushrooms stored at 2.2ºC, being the equivalent of 70 µg $D_2$/100 g FW (Roberts et al., 2008). Considered together, these studies suggest that UV-exposed fresh mushrooms will retain nutritionally relevant amounts of vitamin $D_2$ when refrigerated for one week or less.

Three types of mushroom (button, shiitake and oyster) exposed to a UV-B lamp and then hot-air dried, had relatively good retention of vitamin $D_2$ up to 8 months when stored in dry, dark conditions at 20ºC in closed plastic containers (Sławińska et al., 2016). However, there was a steady loss of vitamin $D_2$ during storage between 8-18 months. In the case of hot-air dried button mushrooms, the vitamin $D_2$ concentration decreased from 14.3 µg/g DM to 9.3 µg/g DM over 8 months, then to 6.9 µg/g DM over the following 10 months.

*6.2. Cooking*

Very few studies have investigated the effect of cooking on the concentration of vitamin $D_2$ in vitamin D-enhanced mushrooms. Following five minutes of frying without oil, two types of wild chanterelle mushrooms retained at least 85% of their raw state content of vitamin $D_2$ concentrations after adjusting for water loss during cooking (Ložnjak & Jakobsen, 2018; P. Mattila, Ronkainen, Lehikoinen, & Piironen, 1999). In button mushrooms with a vitamin $D_2$ content of 19 µg/100 g FW, the retention of vitamin $D_2$ after boiling in water for 20 min, or oven-baking for 10 mins, was 62-67%; for mushrooms pan-fried without oil for 5 min the retention was again high at 88% (Ložnjak & Jakobsen, 2018). This indicates that the duration of cooking and/or cooking method may be important factors in vitamin $D_2$ retention in mushrooms.

## 7. Bioavailability of vitamin $D_2$ from mushrooms

One of the earliest studies to determine the bioavailability of vitamin $D_2$ was from wild chanterelles in the 1990s (Outila, Mattila, Piironen, & Lamberg-Allardt, 1999). In participants with a baseline mean serum 25(OH)D concentration of 38.5 nmol/L, the vitamin $D_2$ in mushrooms increased serum 25(OH)D concentrations in 27 participants as effectively as a vitamin $D_2$ supplement after 3 weeks. Since then, the bioavailability of vitamin $D_2$ from mushrooms has been demonstrated in both rats (Calvo et al., 2013; Jasinghe, Perera, & Barlow, 2005; Koyyalamudi et al., 2009) and humans (Keegan et al., 2013; Mehrotra et al., 2014; Stephensen et al., 2012; Stepien et al., 2013; Urbain, Singler, Ihorst, Biesalski, & Bertz, 2011), and there is evidence that vitamin $D_2$ from mushrooms supports bone health in animal models (Calvo et al., 2013; S. Y. Chen et al., 2015; Jasinghe et al., 2006; G. Lee, Byun, Yoon, Choi, & Jeung, 2009).

The bioavailability of vitamin $D_2$ from mushrooms was assessed in a study of thirty healthy adults who were randomised to receive 2000 IU (50 µg) of supplemental vitamin $D_2$, mushroom vitamin $D_2$, or vitamin $D_3$ for three months (Keegan et al., 2013). Vitamin $D_2$ from mushrooms was as effective as supplemental vitamin $D_2$ in raising and maintaining serum 25(OH)$D_2$ concentrations. Similarly, a five-week study in adults with serum 25(OH)D (combined 25(OH)$D_2$ and 25(OH)$D_3$) concentrations less than 50 nmol/L showed that vitamin $D_2$ from soup made from UV-B irradiated mushrooms improved vitamin D status as effectively as supplemental vitamin $D_2$ (Urbain et al., 2011). However, another study providing UV-irradiated

mushrooms as part of a meal for six weeks increased serum 25(OH)$D_2$ concentrations in participants but serum 25(OH)$D_3$ concentrations decreased. Overall, there was no effect of the UV-irradiated mushrooms on vitamin D status (Stephensen et al., 2012). Although the weight of research evidence indicates that vitamin $D_3$ is more effective than vitamin $D_2$ in raising circulating 25(OH)D concentrations (Tripkovic et al., 2017; Wilson, Tripkovic, Hart, & Lanham-New, 2017), it should also be acknowledged that vitamin $D_3$ is not suited to many vegetarians and that a source of vitamin $D_2$ may be preferred.

**8. Conclusion**

Mushroom consumption is increasing rapidly worldwide, with the production of mushrooms rising from 1 billion kg in 1978 to 27 billion kg in 2012 (an increase in per capita consumption from 0.25 kg to 4 kg) (Royse, 2014). Since mushrooms provide nutritionally relevant amounts of B group vitamins, and the minerals selenium, potassium, copper and zinc, they are a nutritious, low energy-dense food (Feeney, Miller, & Roupas, 2014; Institute of Food Research, 2015). Currently, some larger commercial mushroom farms in the USA, Ireland, the Netherlands and Australia expose fresh mushrooms to UV radiation, generating at least 10 μg $D_2$/100 g FW, therefore, a 100 g serve would provide 50-100% of the daily vitamin D requirements of consumers. Exposing dried mushrooms to UV-B radiation can also generate nutritionally useful amounts of vitamin $D_2$, although this practice is not widespread to date.

It is conceivable that UV-B radiation post-harvest (for fresh mushrooms) or post-drying (for dried and powdered mushrooms) could become standard commercial practice. Sunlight, regular UV lamps and pulsed UV lamps all have the capability to raise the vitamin $D_2$ concentrations to nutritional significance, although pulsed UV lamps may be the most cost-efficient method for commercial production of vitamin D-enhanced mushrooms due to the low exposure time to achieve at least 10 μg/100g FW, often in 1-3 seconds. There is minimal discolouration in mushrooms after pulsed UV treatment, possibly due to the small exposure time of less than 4 s (Kalaras et al., 2012); however, there are many reports of surface discolouration of mushrooms after longer exposures to UV radiation from UV fluorescent lamps (Ko et al., 2008; Koyyalamudi et al., 2009; Mau et al., 1998; Teichmann et al., 2007). Since consumers may be deterred by discolouration in mushrooms, pulsed UV treatment is likely to be preferred by commercial growers.

Vitamin D-enhanced mushrooms contain high concentrations of vitamin $D_2$, which is bioavailable and relatively stable during storage and cooking. Therefore, consumption of vitamin D-enhanced mushrooms could substantially contribute to alleviating the global public health issue of vitamin D deficiency. Further research is warranted to determine the optimal level of UV radiation required to produce a nutritionally useful amount of vitamin $D_2$ in mushrooms, along with optimal storage conditions and cooking methods. The physiological benefits of mushroom-derived vitamin $D_2$ compared with solar-derived vitamin $D_3$, also requires further investigation.

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**2.2. Papers published after the review**

Since the review article was published, there have been other studies on similar themes, although none have changed the sentiment or the conclusions of the review. The following additional relevant information is current to January 2023.

Keflie et al (Keflie, Nölle, Lambert, Nohr, & Biesalski, 2019) placed oyster mushrooms outside in ambient sunlight as the source of UV radiation for up to 16 h on cloudless days at a temperature range of 21 – 34 ºC. The study was conducted during September in Addis Ababa, Ethiopia. The mushrooms were monitored for changes in moisture, texture and colour. The mean vitamin $D_2$ content in mushrooms cut into 1 cm cubes rose from 0 to 73.7 ± 12.9 μg/g DW after 3 h of sun exposure. It was not clear whether the cubes included gills where there may be a higher content of ergosterol (Jasinghe & Perera, 2005). However, the authors reported that only 30 minutes or less of direct sunlight was required for achieving the daily requirement of vitamin D (mean 29 μg/g DW after 30 min) from fresh oyster mushrooms without any noticeable change in colour or texture. Cut mushroom cubes of varying sizes were also exposed to a UV-B lamp up to a dose of 1.5 $J/cm^2$ and it was reported that UV-B lamp exposure was about 10 times more effective than sun exposure at generating vitamin $D_2$.

There have been two further papers on the stability and retention of vitamin $D_2$ when fresh button mushrooms were cooked. Both studies boiled either whole or sliced button mushrooms and found a vitamin $D_2$ retention of 88-92% (Malik, Jan, Haq, Kaur, & Panda, 2022; Salemi et al., 2021). They also reported a high vitamin $D_2$ retention after baking mushrooms. Roasting samples for 8 minutes at 160 ºC resulted in 75% retention (Malik, Jan, Haq, et al., 2022), while Salemi et al. reported a 94% retention after 10 minutes of roasting at an unknown temperature

(Salemi et al., 2021). One reason for the difference might be because the former roasted sliced mushrooms, while the latter roasted whole mushrooms. One of the studies also fried mushrooms in oil for 6 minutes with a 67% retention of vitamin $D_2$ (Malik, Jan, Haq, et al., 2022). The more recent cooking studies are important because that is an area of research where data are lacking.

Both the above studies also observed the effect on vitamin $D_2$ concentration of up to seven days storage (refrigerated, frozen and room temperature). The stability of the vitamin $D_2$ in UV-B-irradiated oyster, shiitake and button mushrooms was observed over five days, refrigerated at either 4 °C or stored at a room temperature of 30 °C (Malik, Jan, Haq, et al., 2022). When button mushrooms were stored at 30 °C, the vitamin $D_2$ concentration peaked at day 3 (83 µg/g FW) before dropping to 25 µg/g FW after five days. When the same mushrooms were refrigerated at 4 °C the vitamin $D_2$ concentration peaked at days 3 and 4 (51 µg/g FW) then dropped to 42 µg/g FW by the end of day 5, suggesting in both cases that vitamin $D_2$ can rise in the three days following UV-irradiation, whether at room temperature or refrigerated, before dropping quickly.

In the study by Salemi et al., button mushrooms were stored at either -14 °C, 4 °C or 25 °C. This resulted in vitamin $D_2$ concentrations being roughly halved from the original level of 14.8 µg/g FW when the mushrooms were either frozen or refrigerated for seven days (Salemi et al., 2021). Measurements were at only 0 and 7 days, therefore it is unknown if there was a rise and fall in that interval, as found in the Malik paper. The vitamin $D_2$ levels dropped 8% (non-significant) when kept at 25 °C for a day.

Two studies confirmed that mushroom-derived vitamin $D_2$ is bioavailable. In the first study human participants daily consumed either a placebo, a vitamin $D_3$ supplement or a UV-irradiated mushroom vitamin $D_2$ powder (25 µg $D_2$) over 12 weeks (Pinto, Merzbach, Willmott, Antonio, & Roberts, 2020). The mushroom-derived vitamin $D_2$ powder was effective in maintaining total serum 25(OH)D and increased serum 25(OH)$D_2$, although at the expense of 25(OH)$D_3$. The authors surmised that UV-treated mushroom powder as a supplement would be affordable and widely applicable to those with low vitamin D status, especially if they want to avoid animal products. The second was an animal study of rats on a vitamin D-deficient diet, where the rats were given a supplement of UV-irradiated shiitake mushroom powder (Won, 2019). The study showed that the vitamin $D_2$ was bioavailable and increased bone mineral density of the femur.

The recent studies have added more support to the potential role of button mushrooms as a good source of vitamin D. They demonstrate a good retention of vitamin $D_2$ after cooking and short-term storage. They also confirm that the vitamin $D_2$ from mushrooms is bioavailable, giving confidence to the view that sufficient vitamin D can survive drying, cooking, and digestion to play a positive role in bone physiology and other associated functions.

Finally, a theoretical paper evaluated the nutrient intake of an individual who has an additional serve (84 g) of mushrooms to their usual diet (Fulgoni & Agarwal, 2021). There was an increase in key nutrients such as fibre (5%), selenium (13%), riboflavin (14%) and niacin (13%), without any impact on energy (kJ), carbohydrate or sodium. If the mushrooms were exposed to UV radiation to provide 5.0 μg vitamin $D_2$ per serve, then vitamin D intake doubled.

There are still some gaps that need to be addressed before we can be confident in offering vitamin D-enriched dried mushrooms to the consumer. The ideal range of exposure to UV radiation of each type of common edible mushroom to generate nutritionally useful levels of D-vitamers needs to be established. This includes oyster, shiitake, enoki and button mushrooms and applies to both fresh and dried mushrooms. Some countries state the recommended upper level of daily intake of vitamin D in their government nutrient requirement guidelines, and it is important that concentrations in mushrooms do not exceed these, even though the recommended maximum levels are not indicative of a dangerous level.

**Chapter 3: Effect of air-drying on the generation of vitamin $D_2$ and 25-hydroxyvitamin $D_2$ by pulsed UV irradiation in button mushroom (*Agaricus bisporus*)**

**Objective 2:** Verify the method for the extraction of D vitamers from a mushroom matrix and determine the ideal sequence of irradiation and air-drying of mushrooms and the generation of D vitamers.

The study was presented as a poster prior to publication at the International Conference of Food Analysis, 16-17 November 2021 (see Appendix I, p 92).

Following is the final published study (*Journal of Food Composition and Analysis* 115: 105034. doi: 10.1016/j.jfca.2022.105034):

## Effect of air-drying on the generation of vitamin $D_2$ and 25-hydroxyvitamin $D_2$ by pulsed UV irradiation in button mushroom (*Agaricus bisporus*)

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**Abstract**

Fresh and dried mushrooms naturally generate vitamin $D_2$ when exposed to ultraviolet (UV) radiation. Vitamin $D_2$ and 25-hydroxyvitamin $D_2$ (25(OH)$D_2$) concentrations were compared in dried button mushrooms (*Agaricus bisporus*) exposed to pulsed UV radiation either before or after air-drying. A further aim was to assess the effect of air-drying on the generation of D vitamers. Fresh button mushrooms were irradiated (Irr) with a total of 200 mJ/cm$^2$ pulsed UV radiation before (Irr/AD) or after (AD/Irr) being air-dried (AD). A third group of fresh button mushrooms was irradiated but not air-dried (Irr). Control mushrooms were fresh and untreated. The D vitamers were quantified in freeze-dried samples using triple quadrupole mass spectrometry. Irr/AD mushrooms had more than double the concentration of vitamin $D_2$ than

AD/Irr mushrooms (9.5 µg/g dry weight (DW) vs 4.6 µg/g DW). However, Irr mushrooms contained 6.3 µg/g DW. The concentration of 25(OH)$D_2$ in Irr mushrooms was 0.05 µg/g DW, while 0.14 µg/g DW was detected in Irr/AD mushrooms. There was no detectable 25(OH)$D_2$ in control mushrooms, nor in AD/Irr mushrooms. The sequence of irradiating and drying mushrooms was a key factor in generating vitamin $D_2$.

**Key words:** *Agaricus bisporus*; mushroom; vitamin $D_2$; 25-hydroxyvitamin $D_2$; pulsed ultraviolet radiation; air-drying

**Abbreviations:**

UV: ultraviolet; 25(OH)$D_2$: 25-hydroxyvitamin $D_2$; LC-QQQ: Liquid chromatography with triple quadrupole tandem mass spectrometry; LOD: limit of detection

## 1. Introduction

The natural dietary sources of vitamin D are mainly animal products, such as fish, meat and egg yolk. In many countries vitamin D intakes are low, despite some foods being fortified with vitamin D (Bailey, 2010; L.J. Black et al., 2015; L. J. Black, Walton, Flynn, & Kiely, 2013; Dunlop et al., 2023; Vatanparast, 2010). There is a high prevalence of vitamin D deficiency in Australia and globally (L. J. Black et al., 2021; K.D. Cashman, 2015; Horton-French, Dunlop, Lucas, Pereira, & Black, 2019, 2021; Malacova et al., 2019). Ultraviolet (UV) irradiated mushrooms can generate high amounts of vitamin $D_2$, and are the only commonly consumed non-animal, natural source of vitamin D, making them an important dietary source of vitamin D, especially for vegetarians and vegans (Cardwell et al., 2018; Jasinghe & Perera, 2006; Kalaras et al., 2012).

*Agaricus bisporus* (common button mushroom) is one of the most highly consumed mushroom species in the western world, along with *Lentinula*, *Pleurotus* and *Auricularia* (Royse et al., 2017). The worldwide market for dried mushrooms is not clear, although the United Nations Food and Agriculture Organisation has provided imputed data on the dried mushroom supply in selected countries up to 2019 (Food and Agriculture Organisation, 2020). Wild and cultivated dried mushrooms analysed from the Chinese market all contained vitamin $D_2$ (average 16.9 µg $D_2$/g dried mushroom; range 7.7-25.0 µg $D_2$/g), most likely from sun exposure during drying and packing (Huang et al., 2016).

Ergosterol is readily converted to pre-vitamin $D_2$ when exposed to a source of UV-B (280-315 nm) or UV-C (100-280 nm) radiation, for example, solar radiation (UV-B), a conventional UV lamp (UV-B and/or C) or a pulsed UV lamp (UV-B and UV-C), with UV-B wavelengths being the most efficient at generating pre-vitamin $D_2$ (Jasinghe & Perera, 2006; Wu & Ahn, 2014). Pre-vitamin $D_2$ is then thermally isomerised in a temperature-dependent process to ergocalciferol (vitamin $D_2$) (Keegan et al., 2013). Previous studies (Kalaras et al., 2012; Koyyalamudi et al., 2011; Kristensen et al., 2012; Teichmann et al., 2007; Urbain & Jakobsen, 2015) have shown that exposing fresh button mushrooms to sunlight, a conventional UV-B or UV-C lamp or a pulsed UV lamp (200-400 nm) can generate amounts of vitamin $D_2$ that exceed the daily requirements of vitamin D (15-20 μg) (Institute of Medicine, 2011) in a single serve (100g).

Pulsed UV radiation is commonly used in commercial settings because, within seconds, the concentration of vitamin $D_2$ in mushrooms increases to concentrations that take conventional UV lamps (non-pulse) several minutes to achieve (Cardwell et al., 2018). To our knowledge, only four previous studies have involved exposing mushrooms to pulsed UV radiation prior to quantifying vitamin $D_2$. Two of these studied fresh button mushrooms (Kalaras et al., 2012; Koyyalamudi et al., 2011), another study used varieties of fresh and powdered *Pleurotus* (oyster) mushrooms (S.-Y. Chen et al., 2015), and the final study worked on freeze-dried powdered *Lentinula edodes* (shiitake) (Chien et al., 2017). All studies concluded that pulsed UV radiation is an efficient method to generate high concentrations of vitamin $D_2$ in fresh and powdered mushrooms, although none of them exposed dried button mushrooms to pulsed UV radiation.

To our knowledge, this is the first study to assess the effect of pulsed UV radiation on the generation of vitamin $D_2$ and 25-hydroxyvitamin $D_2$ (25(OH)$D_2$) in dried button mushrooms. The main aim of the current study was to compare the concentrations of vitamin $D_2$ and 25(OH)$D_2$ in dried button mushrooms exposed to the same dose of pulsed UV radiation either before (Irr/AD) or after (AD/Irr) air-drying. In addition, to assess the effect of air-drying separately on the generation of D vitamers, concentrations of D vitamers in irradiated dried mushrooms were compared to those found in irradiated fresh mushrooms (Irr) exposed to the same dose of pulsed UV radiation.

The potential effect of the sequence of irradiating and drying on the concentration of vitamin $D_2$ could provide useful information to growers about the optimal sequence of drying and irradiating when producing vitamin D-enhanced dried mushrooms.

## 2. Materials and methods

### *2.1. Mushroom samples*

Sixteen packages each containing about 200 g of fresh button mushrooms (*Agaricus bisporus* (J.E. Lange) Imbach 1946) with diameters 5-6 cm were collected directly from a farm (Costa Mushrooms) in Perth, Western Australia. The mushrooms were transported by road (~30 km) in light-sealed, cooled, insulated boxes to the laboratory. There was no opportunity for accidental exposure to a source of UV radiation, such as sunlight. The mean weight of each mushroom was 27.8 g (standard deviation (SD) 2.2). The skirt over the gills remained intact. Any residual compost was brushed from the surface of the mushrooms. Mushrooms were refrigerated at 4 ºC and treatment began within 24 h of harvesting. All mushrooms were kept at room temperature (23 ºC) for 2 h prior to treatment. The mushroom packages were randomly placed in one of four groups and treated as follows: 1) control i.e. no UV exposure (Section 2.2); 2) Irr (Section 2.3); 3) Irr/AD (Section 2.4); and 4) AD/Irr (Section 2.5). The radiation dose was calculated from a preliminary trial to determine a suitable dose that would provide at least the recommended daily requirement of vitamin D (15-20 μg) (Institute of Medicine, 2011) in 100 g of fresh mushrooms.

### *2.2. Preparation of control samples*

Mushroom samples (200 g; n=4) were sliced to 2-3 mm thickness, vacuum sealed and refrigerated at 4 ºC.

### *2.3. Preparation of Irr mushroom samples*

Each 200 g sample of mushrooms (n=4) was arranged in a single layer with skirted gills towards the UV radiation source. The distance from the surface of the mushrooms to the lamp was 10 cm. They were subjected to five pulses of UV radiation (260-400 nm) from a pulsed light system (Wek-tec XeMaticA-2L, Germany), providing an average total dose of 200 $mJ/cm^2$ as measured by a radiometer in the 215-350 nm spectrum (International Light Technologies

ILT800 CureRight, USA). After irradiation, they were sliced to 2-3 mm thickness, vacuum sealed and refrigerated at 4 ºC in preparation for freeze-drying within 24 hours.

*2.4. Preparation Irr/AD mushroom samples*

Mushroom samples (200 g; n=4) were irradiated as per Section 2.3. After two hours they were placed in a single layer on shelves and air-dried in a convection oven for 18 h at 60 ºC (Contherm Thermotec 2000, Contherm Scientific Ltd, New Zealand), which is the most common temperature used commercially (Argyropoulos, Heindl, & Müller, 2011). Fresh button mushrooms naturally have a high moisture content. Others have reported a 90% loss of fresh weight after air-drying (Gallotti & Lavelli, 2020; Pedrali, Gallotti, Proserpio, Pagliarini, & Lavelli, 2020). The current study showed a consistent loss of 90.2-90.3% after air-drying (Table 1). Air-drying reduced the diameter of the mushrooms from 5-6 cm to 3-4 cm.

*2.5. Preparation of AD/Irr mushroom samples*

Mushroom samples (200 g; n=4) were air-dried as in Section 2.4 and then irradiated approximately one hour later, as in Section 2.3, although the distance of the dried mushrooms from the lamp increased from 10 to 12 cm due to shrinkage during the drying process.

*2.6. Freeze drying and packaging*

All samples from sections 2.2 to 2.5 were vacuum sealed and refrigerated at 4 ºC to be lyophilised within 24 h in a freeze dryer (Christ Alpha 1-2 LD plus, Germany) at -30 ºC and 37 Pa for the following times: 68 h for C; 68 h for Irr; 24 h for Irr/AD; 24 h for AD/Irr. Lyophilised samples were crushed with a mortar and pestle, then milled to a fine powder with a blade grinder (KitchenAid, Australia). All freeze-dried samples were packed in sealed, labelled 50 mL sample jars and stored at -20 ºC. The freeze-dried weight was taken to be the dried weight for calculations of D vitamer concentrations as no further weight loss occurred with further drying. The moisture loss after freeze-drying was consistent between samples (91.0-91.7%, Table 1).

**Table 1. Concentrations of D vitamers in mushrooms in the control group and three treatment groups**

| Treatment[2] | Fresh weight, g | Air-dried weight, g | % weight loss[3] | Freeze dried weight, g | % total weight loss[4] | $D_2$ µg/g DW | 25(OH)$D_2$ µg/g DW |
|---|---|---|---|---|---|---|---|
| Control (fresh, untreated) | 202.0 ± 4.4 (196.8-207.0) | N/A | N/A | 17.1 ± 1.7 (15.8-19.5) | 91.6 ± 0.8 (90.4-92.2) | <LOQ[1] | <LOQ[1] |
| Fresh, irradiated (Irr) | 211.8 ± 8.1 (202.6-219.1) | N/A | N/A | 17.6 ± 1.2 (17.0-19.2) | 91.7 ± 0.5 (91.2-92.2) | 6.3 ± 0.9 (5.5-7.7) | 0.05 ± 0.00[b] (0.04-0.06) |
| Fresh, irradiated then air-dried (Irr/AD) | 201.9 ± 2.4 (199.3-204.5) | 19.7 ± 0.2 (19.5-19.9) | 90.2 ± 0.2 (90.0-90.4) | 17.8 ± 0.5 (17.2-18.5) | 91.2 ± 0.2 (90.9-91.4) | 9.5 ± 1.4[a] (7.7-10.9) | 0.14 ± 0.03[b] (0.11-0.17) |
| Fresh, air-dried then irradiated (AD/Irr) | 201.5 ± 3.9 (198.0-206.1) | 19.6 ± 1.5 (17.9-21.2) | 90.3 ± 0.8 (89.3-91.2) | 18.3 ± 0.9 (17.2-19.3) | 91.0 ± 0.5 (90.3-91.6) | 4.6 ± 0.1[a] (4.5-4.7) | <LOQ[1] |

Data presented as mean ± SD. The range of the measurements are shown in parentheses.
DW, dry weight; N/A, not applicable; SD, standard deviation
[1] LOQ <0.001 µg/g
[2] Each sample 200 g; four repetitions of each treatment
[3] Comparison of air-dried weight to fresh weight
[4] Comparison of freeze-dried weight to fresh weight
[a] Statistically significant difference ($p < 0.05$) between Irr/AD and AD/Irr
[b] Statistically significant difference ($p < 0.05$) between Irr and Irr/AD

*2.7. Transport of samples for analysis*

The freeze-dried samples were placed in insulated, light-sealed 50 mL sample jars with commercial frozen cooling bricks, then air-freighted overnight to the National Measurement Institute (NMI), Melbourne, Australia. Upon arrival at NMI all samples were kept frozen at -20 ºC and were analysed for D vitamers within one month.

*2.8. Analysis of D vitamers*

The standard preparation, saponification and extraction procedures were conducted in a laboratory illuminated with yellow fluorescent light (560-590 nm). Freeze-dried mushroom powder (500 mg) was added to a 50 mL screw top Falcon® plastic centrifuge tube before adding 1000 mg of the antioxidant sodium ascorbate and 10 mL milli-Q water conforming to Type 1 international water specifications. The resultant mixture was vortexed to ensure even distribution before the addition of the internal standard of vitamin $D_2$-$^2H_3$ and 25(OH)$D_2$-$^2H_3$ (IsoSciences, Philadelphia, USA). Ethanol (30 mL) and milli-Q water were added to a total volume of 50 mL. The tubes were then capped and hand-shaken, followed by the addition of 2.0 g potassium hydroxide. The remaining headspace of the Falcon® tube was flushed with nitrogen gas to dispel oxygen and limit potential oxidation during saponification. The tube was immediately capped and placed laterally in a shaker bath at 25 ºC for 18 h overnight for saponification.

The tubes were centrifuged at 3750 rpm for 90 s, which is a *g* force of 1887, based on a radius of 12 cm (Pro-Analytical, Centurion Scientific Ltd, United Kingdom). A 10 mL aliquot of the supernatant was pipetted onto diatomaceous earth extraction Chem Elut 10 mL unbuffered cartridges (Agilent Technologies, Santa Clara, USA). After 15 minutes, 60 mL of petroleum ether (BP 40-60 ºC) were added to the extraction cartridges and the resultant eluent was evaporated with nitrogen gas in a Dionex SE500 Gas Evaporative System (Thermo Scientific, USA).

One mL *n*-heptane was added to each sample and vortexed. The final volume was transferred to LC vials (1.8 mL, glass, screw-neck with 400 µL flat-bottomed glass inserts, Waters Corporation, USA) and evaporated before adding 800 µL of 4-phenyl-1, 2, 4-triazoline-3, 5-dione (PTAD) at 2 µg/µL. The samples were allowed to form vitamin D-PTAD derivatives for

10 min, before 200 µL of milli-Q water were added to stop the derivatisation. Of the final volume, 400 µL were pipetted into glass liquid chromatography (LC) vials. The injection volume of 10 µL was analysed by liquid chromatography coupled with triple quadrupole (LC-QQQ), tandem mass spectrometer (Agilent Technologies, 1290 Infinity Series LC System/6460 Triple Quad LC-MS fitted with a Jet Stream ESI source in positive ion mode). To ensure that only one vitamer-PTAD adduct was formed, 5 mM of methylamine were added to the mobile phase. The LC chromatographic column was a Supelco Ascentis Express C18, 15 cm x 3mm, 2.7 $\mu$m (Cat #53816-U, Sigma-Aldrich, Merck, Germany). Each sample was analysed in duplicate and the average of the two results was calculated for each of the four repetitions of the control and each treatment.

**Table 2**. Analytical validation of the recovery of spiked mushroom with D vitamers of 50 to 500 μg/100g in the mushroom matrix

| **Spiking concentration** (μg /100 g) | **Vitamin $D_2$** recovery % ± SD | **25(OH)$D_2$** recovery % ± SD | **Vitamin $D_3$** recovery % ± SD | **25(OH)$D_3$** recovery % ± SD |
|---|---|---|---|---|
| 50 | 99 ± 5 | 106 ± 5 | 91 ± 5 | 89 ± 4 |
| 100 | 99 ± 9 | 107 ± 9 | 95 ± 9 | 92 ± 9 |
| 250 | 108 ± 7 | 112 ± 8 | 101 ± 7 | 100 ± 7 |
| 500 | 102 ± 3 | 104 ± 5 | 97 ± 6 | 94 ± 4 |

SD, standard deviation; 25(OH)$D_2$, 25-hydroxyvitamin $D_2$; 25(OH)$D_3$, 25-hydroxyvitamin $D_3$. Seven repetitions at each level.

**Table S1.** Gradient profile with mobile phase 'A' 0.1% formic acid and 5mM methylamine in milli-Q water and mobile phase 'B' 0.1% formic acid and 5mM methylamine in methanol

| **Time (minutes)** | **A %** | **B %** | **Flow mL/min** |
|---|---|---|---|
| 0.00 | 20 | 80 | 0.6 |
| 1.00 | 20 | 80 | 0.6 |
| 13.00 | 3 | 97 | 0.6 |
| 13.01 | 0 | 100 | 0.6 |
| 17.00 | 0 | 100 | 0.6 |
| 17.01 | 20 | 80 | 0.6 |

*2.9. Verification of the analytical method for mushrooms*

To determine if the method of analysis of D vitamers at the range of quantitation was influenced by the mushroom matrix, samples spiked with known concentrations of D vitamers were analysed and the measured amount was compared to the theoretical values (Table 2). The method was developed from published methodologies as follows: the extraction method was adapted (Jones & Makin, 2000; Strobel, Buddhadasa, Adorno, Stockham, & Greenfield, 2013) and the detection and quantification principle was adapted from Jäpelt and colleagues (Jäpelt, Silvestro, Smedsgaard, Jensen, & Jakobsen, 2013). This confirmed the use of this method for the determination of D vitamers in the mushroom matrix for concentrations up to 500 µg/100 g, with a measurement uncertainty of ± 23%.

Fresh button mushrooms (776 g) were collected from a local mushroom farm and transported them under identical conditions as for the current study. The mushrooms were sliced to 2-3 mm thickness, freeze-dried for 44 h (Christ Alpha 1-2 LD plus, Germany), crushed with a mortar and pestle and milled to a fine powder, resulting in a final weight of 62 g, indicating a 92% reduction in weight.

Due to practical constraints on the usage of vitamer standards, sample analysis weight varied with spiking level. For the control (no spiking) and 50 µg/100 g spiking, the sample analysis weight was 500 mg. For 100, 250 and 500 µg/100 g spiking the sample analysis weights were 250, 100 and 50 mg respectively (spiking of 0.5 mL of 500 ng/mL mixed standard per analysis). The freeze-dried mushroom samples were spiked with D vitamers at four spiked analyte concentrations per 100 g of sample (50 µg, 100 µg, 250 µg, and 500 µg), with seven replicates at each spike concentration. The spiking standards were vitamin $D_2$, 25-hydroxyvitamin $D_2$ (25(OH)$D_2$), vitamin $D_3$ and 25-hydroxyvitamin $D_3$ (25(OH)$D_3$). The internal standards were deuterated vitamin $D_2$, deuterated 25(OH)$D_2$, carbon 13 vitamin $D_3$ and carbon 13 25(OH)$D_3$ (IsoSciences, USA).

For the calibration, additions of 200 µL of internal standard (10 ng/mL), 50, 100, 150, 200, and 300 µL of the working calibration standard (10 ng/mL), and 50, 100, 150, and 200 µL of the mixed calibration standard (100 ng/mL) were evaporated with nitrogen gas at 30 ºC in a Ratek DBH30D evaporator. The standards were re-dissolved in 400 µL of 2.0 mg/mL PTAD solution, mixed and allowed to derivatise for 10 minutes. The process was halted with the addition of 100 µL of milli-Q water followed by vortex mixing, then analysed by method VL454 at the National

Measurement Institute, Australia (gradient profile in Table S1). The method was approved in 2017 by the National Association of Testing Authorities, Australia. (National Measurement Institute, (2017). VL454 Determination of vitamin D and 25-hydroxyvitamin D by LC-QQQ, 1.0 ed.).

### *2.10. Statistical analysis*

For each treatment group, the results from the four 200 g samples were averaged for vitamin $D_2$ and 25(OH)$D_2$ concentrations. Two-sample Wilcoxon rank-sum tests were used to identify significant differences of $p<0.05$ for differences between vitamin $D_2$ concentrations in Irr/AD mushrooms and AD/Irr mushrooms; and for 25(OH)$D_2$ concentrations in Irr mushrooms and Irr/AD mushrooms, the groups with detectable 25(OH)$D_2$.
Stata Statistical Software (Version 14.2, StataCorp, USA) was used for statistical analysis.

## **3. Results and discussion**

### *3.1 Concentrations of vitamin $D_2$ in mushroom*

The concentrations of vitamin $D_2$ and 25(OH)$D_2$ in the control samples were less than 0.01 and 0.02 µg/g DW respectively (the limit of detection), demonstrating that there was no exposure of mushrooms to UV radiation at the farm or during transportation to the laboratory. Table 1 shows the mean, SD and range for each of the four groups (control group and three treatment groups) including initial weights, weights prior to irradiation and water loss associated with air and/or freeze drying. Irr/AD mushrooms generated 2.1 times the vitamin $D_2$ content compared to AD/Irr mushrooms (Table 1). The difference was statistically significant ($z = 2.3$, $p<0.05$) indicating that irradiating mushrooms prior to, rather than after, air-drying was more efficient at generating vitamin $D_2$.

Commercial packs of imported dried mushrooms from China report a serving size between 10 and 30 g. There is no agreed serving size for dried mushrooms. Using 20 g as a serving size, the current data showed that a serve of Irr/AD mushrooms would provide approximately 172 µg $D_2$, whereas a serve of AD/Irr mushrooms would provide approximately 86 µg $D_2$. Both concentrations are more than the recommended daily intake of vitamin D in Australia (5-15 µg in adults) (National Health and Medical Research Council, 2006), Europe (15 µg) (EFSA, 2017), the Nordic countries (10-20 µg) (Nordic Council of Ministers, 2014) and the USA (15-20 µg) (Institute of Medicine, 2011).

It is not clear why irradiating mushrooms prior to, rather than after, drying would be a more efficient process for generating vitamin $D_2$. However, air-drying prior to irradiation may disrupt the ability of ergosterol to convert to vitamin $D_2$. A threshold minimal fluid amount may be required for optimal conversion of ergosterol to vitamin $D_2$ by UV radiation and, as the water content drops, the rate of vitamin $D_2$ generation may diminish. One study found that the amount of ergosterol converting to vitamin $D_2$ in shiitake mushrooms reduced as the moisture content dropped below 70% (Perera et al., 2003). In the current study, the AD/Irr mushrooms had a smaller surface area than their fresh counterpart (9.6 $cm^2$ vs 23.8 $cm^2$ based on an average circumference of 3.5 cm and 5.5 cm, respectively), and were 2 cm further from the radiation source due to shrinkage during the air-drying process. This means that they would have received a smaller total UV dose by 11% based on the radiometer measurements performed at the two different distances from the irradiation lamp. This may have partially contributed to the lower vitamin $D_2$ content in mushrooms that were dried before irradiating compared to irradiated before drying.

Irr/AD mushrooms also produced 1.5 times more vitamin $D_2$ concentration than Irr mushrooms (Table 1). Once fresh mushrooms are UV-irradiated, some of their abundant ergosterol is converted to pre-vitamin $D_2$, then ambient heat is required to isomerise pre-vitamin $D_2$ to vitamin $D_2$. This temperature-dependent conversion is likely due to a non-enzymatic membrane-enhanced catalytic mechanism similar to that which occurs in human skin (Keegan et al., 2013; Tian, Chen, Matsuoka, Wortsman, & Holick, 1993). Thirty internal temperature measurements were carried out on whole mushrooms via the stipe every 15 minutes during air-drying at 60 ºC. The internal temperature was 25 ºC at 15 min, 36 ºC at 30 min, then stabilised at 42 ºC from 45-150 min. Therefore, when the mushrooms were placed in the air-dryer at 60 ºC after irradiation, the continued application of heat may have promoted the conversion of residual pre-vitamin $D_2$ to vitamin $D_2$ to a greater extent than would occur at room temperature (23 ºC in the laboratory) in fresh, irradiated mushrooms.

There was no statistically significant change in vitamin $D_2$ content in a study of whole button mushrooms exposed to a UV-B lamp before and after air-drying for 20 h at 40-60 ºC (Sławińska et al., 2016). The drying time and temperature (20 h; 40-60 ºC) were very similar to the current study (18 h; 60 ºC). However, one difference is that the mushrooms in that study were sliced prior to hot-air drying; in the current study, mushrooms remained whole during drying. Another critical difference between the studies was the amount of UV irradiation, whereby the

mushrooms in the current study received half the dose (i.e., 200 as opposed to 411 mJ/cm$^2$); and the fresh, irradiated mushrooms produced approximately half the concentration of vitamin $D_2$ (6.3 as opposed to 13.1 µg/g DW).

A further potential factor influencing the vitamin $D_2$ concentrations of mushrooms when air-dried is the size of the whole mushroom. The internal temperature of larger diameter whole mushrooms rises at a slower rate than in smaller diameter or sliced mushrooms, potentially allowing more time for pre-vitamin $D_2$ to convert to vitamin $D_2$ before reaching a threshold temperature at which no further conversion occurs.

*3.2 Concentrations of 25(OH)$D_2$*

25-hydroxyvitamin $D_2$ was detected in Irr mushrooms, and in Irr/AD mushrooms. Comparing the dry weight concentrations, Irr mushrooms had lower concentrations of 25(OH)$D_2$ compared to Irr/AD mushrooms ($z = 2.46$, $p<0.05$). These results suggest that air-drying mushrooms before irradiating inhibits their ability to generate 25(OH)$D_2$. The production of 25(OH)$D_2$ in mushrooms is possibly due to the presence of cytochrome P450 enzymes. In humans, the enzyme CYP2R1 (a liver microsomal cytochrome P450 enzyme) is required to hydroxylate ergocalciferol to 25-hydroxyergocalciferol (Cheng, Levine, Bell, Mangelsdorf, & Russell, 2004). As mushrooms contain many P450 enzymes, it is likely that a very similar enzyme performs the hydroxylating function (van den Brink, van Gorcom, van den Hondel, & Punt, 1998). One study found that the bacterial cytochrome P450 enzyme CYP109E1 had a strong affinity for vitamin $D_2$, especially in hydroxylating at the C24 and C25 positions (Putkaradze, König, Kattner, Hutter, & Bernhardt, 2020). Liver P450 denatures at about 50 ºC (Anzenbacher, Hudeček, & Stružinsky, 1982), while most other regular P450s denatures between 50 ºC and 60 ºC (Z. Liu et al., 2018). Hence, air-drying at 60 ºC may have denatured the P450 enzymes in mushrooms and inhibited the generation of 25(OH)$D_2$ via irradiation. Although the nutritional importance of low concentrations of 25(OH)$D_2$ in mushrooms may be minor, the current study suggests that the sequence of irradiation and drying affects the generation of this D vitamer. There was no detectable 25(OH)$D_2$ in the control samples nor in AD/Irr mushrooms. The only other study to analyse the 25(OH)$D_2$ in button mushrooms also did not quantify any in fresh, non-irradiated mushrooms (P. H. Mattila et al., 1994).

Vitamin $D_2$ and 25(OH)$D_2$ were only analysed at a single irradiation dose (200 mJ/cm$^2$), and air-drying temperature of 60 ºC, after a specific time (18 h), with a specific orientation

(underside facing the lamp). It is likely that a higher irradiation dose would have resulted in a higher concentration of D vitamers (Ko et al., 2008). Thus, determining the ideal irradiation dose, drying time and temperature to promote the maximum concentration of D vitamers would require a wide range of different treatment conditions. Only mushrooms of a size (diameter) and maturity (with gills covered) commonly sold at retail outlets were used in the current study. Using mushrooms of different sizes and levels of maturity may also have a bearing on the concentration of D vitamers. More mature mushrooms have their gills exposed, and this would likely create more vitamin $D_2$ at a faster rate (Krings & Berger, 2014; Perera et al., 2003). Ergosterol (pro-vitamin $D_2$) is abundant in button mushrooms, present in levels of 4500-6500 µg/g DW, in the magnitude of 1000 times the concentration of vitamin $D_2$ in this study (Gallotti & Lavelli, 2020; Hammann, Lehnert, & Vetter, 2016; P.H. Mattila et al., 2002; K. M. Phillips et al., 2011). Two studies specifically noted that there is little difference in the ergosterol levels between control and UV irradiated button mushrooms (Gallotti & Lavelli, 2020; Simon et al., 2011). For this reason, the ergosterol levels were not measured in the current study as it is not a limiting factor in the generation of vitamin $D_2$.

## 4. Conclusion

Exposing mushrooms to pulsed UV radiation before air-drying generated more vitamin $D_2$ and $25(OH)D_2$ than exposing them after air-drying. The D vitamers were generated in concentrations that reached or exceeded the recommended daily dietary amounts in a 20 g serve of dried mushrooms. These findings could be especially important considering the high prevalence of vitamin D deficiency globally. Commercially dried, UV-irradiated mushrooms have the potential to be a valuable, long shelf life, non-animal source of vitamin D in a food supply that is largely lacking in vitamin D. Mushroom producers supplying vitamin D-enriched dried mushrooms through UV irradiation could consider the enhanced efficacy of exposing mushrooms to UV radiation before, compared to after, the drying process.

## CRediT authorship contribution statement

**Glenn Cardwell:** Conceptualisation, Methodology, Method verification, Formal analysis, Writing – original draft. **Lucinda Black:** Supervision, Writing – review & editing, Project administration. **Janet Bornman:** Supervision, Writing – review & editing. **Anthony James:** Supervision, Writing – review & editing. **Alison Daly**: Statistics. **Norbert Strobel:** Methodology, Method verification, Resources. **Jette Jakobsen:** Writing – review & editing.

**Declaration of Competing Interest** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data availability**

Data will be made available on request.

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**Chapter 4: Vitamin $D_2$ and 25-hydroxyvitamin $D_2$ retention in pulse UV-irradiated dried button mushrooms (*Agaricus bisporus*) after 3, 6 and 12 months of storage**

**Objective 3:**

To analyse the retention of D vitamers in pulse UV-irradiated, air-dried mushrooms when stored for up to 12 months in conditions normally experienced in a southern Australian household pantry.

As it is very convenient to store dried mushrooms in households until required, the next study aimed to assess the retention of D vitamers in UV-exposed dried mushrooms over 12 months of storage. The samples were first UV-irradiated then air-dried as that was the most efficient sequence as shown in the previous chapter. It was assumed that if there were still nutritionally useful concentrations of D vitamers remaining after 12 months, then vitamin D-enhanced dried mushrooms would be a viable option as a non-animal dietary source of vitamin D. One previous study did demonstrate good retention of vitamin $D_2$ after exposure to a conventional UV lamp (Sławińska et al., 2016). This was the first study using pulsed UV radiation and assessment of the retention of both vitamin $D_2$ and 25(OH)$D_2$ in dried mushrooms.

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## Vitamin $D_2$ and 25-hydroxyvitamin $D_2$ retention in pulse UV-irradiated dried button mushrooms (*Agaricus bisporus*) after 3, 6 and 12 months of storage

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**Abstract:** Fresh mushrooms exposed to a source of ultraviolet (UV) radiation prior to drying generate high concentrations of vitamin $D_2$. There are few studies on the stability of vitamin $D_2$ in dried mushrooms during long-term storage. The aim of this study was to determine the retention of D vitamers in mushrooms that were pulse UV-irradiated, then air-dried, and stored

for up to 12 months. Fresh button mushrooms (*A. bisporus*) were exposed to pulsed UV radiation (dose 200 $mJ/cm^2$, peak of 17.5 $W/cm^2$), air-dried in a convection oven (60 ºC for 18 h) and vacuum-sealed before being stored at room temperature (18-26 ºC, 40-65% humidity) in a container protected from any further UV radiation. After storage, the D vitamers were quantified in freeze-dried samples using triple quadrupole mass spectrometry. There was 100%, 93% and 58% retention of vitamin $D_2$ and 88%, 71% and 68% retention of 25-hydroxyvitamin $D_2$ (25(OH)$D_2$) after 3-, 6- and 12-months of storage, respectively. Compared to baseline, the D vitamer concentration was statistically significantly lower ($p<0.05$) at 6 and 12 months for 25(OH)$D_2$ and at 12 months for vitamin $D_2$. There was still sufficient vitamin $D_2$ remaining after 12 months to provide at least 100% of daily dietary vitamin D requirements in a 20 g serving of UV-irradiated, dried mushrooms.

**Keywords:** *Agaricus bisporus*; 25-hydroxyvitamin $D_2$; air-drying; mushroom; pulsed ultraviolet radiation; storage; vitamin $D_2$

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## 1. Introduction

Vitamin D deficiency is prevalent worldwide, partly because there is low consumption of dietary vitamin D (Ahmed, Ng, & M.R., 2021; Dunlop et al., 2023; Herrick et al., 2019; Lips et al., 2019), and partly because of cultural norms or measures to reduce sun exposure to potentially damaging ultraviolet (UV) radiation (Amrein et al., 2020; K. D. Cashman, 2022). Very few foods are a good source of vitamin D, the exceptions being meat, fish and egg yolk, and foods that have been fortified with vitamin D. Mushrooms generate vitamin $D_2$ and 25-hydroxyvitamin $D_2$ (25(OH)$D_2$) when exposed to ultraviolet (UV) radiation such as solar radiation or UV lamps, with UV-B radiation being the most effective wavelength range (Cardwell et al., 2018; Jasinghe & Perera, 2006; Wu & Ahn, 2014). The ergosterol (pro-vitamin $D_2$) in mushrooms first becomes pre-vitamin $D_2$ after exposure to UV radiation, then it is subsequently thermally isomerised to ergocalciferol (vitamin $D_2$) (Keegan et al., 2013). Wild mushrooms that have been exposed to sunlight while growing or drying will naturally generate vitamin $D_2$ (Keflie et al., 2019; P. H. Mattila et al., 1994; Teichmann et al., 2007). Commercial dried mushrooms are normally sun-dried or hot-air dried, the former resulting in production of vitamin $D_2$ (Argyropoulos et al., 2011; Huang et al., 2016). UV-exposed dried mushrooms can be a convenient and valuable source of vitamin D, especially for those wishing to limit sun exposure or avoid animal-derived dietary products (Nölle et al., 2016; Rangel-Castro et al., 2002; Sławińska et al., 2016).

The Food and Agricultural Organization of the United Nations has estimated annual production for dried mushrooms by country (Food and Agriculture Organisation, 2020). Commercial dried mushrooms are often labelled with a shelf life of 12-18 months after packaging. Some countries permit dried mushrooms to be sold for up to 24 months after packaging, although there may be significant deterioration in quality and antioxidant activity beyond 12 months of storage (Jaworska, Pogoń, Bernaś, & Skrzypczak, 2014).

The short-term retention of vitamin $D_2$ has been studied in fresh mushrooms and in the longer term with frozen, powdered or dried mushrooms. The level of retention depends on the storage conditions. The retention of vitamin $D_2$ in UV-irradiated fresh mushrooms when refrigerated over periods of up to 14 days have showed varying results, with losses up to 50% (Guan et al., 2016; Kalaras et al., 2012; Malik, Jan, Haq, et al., 2022; Salemi et al., 2021). Wild mushrooms retained virtually all their vitamin $D_2$ after being frozen for 9 months (P. Mattila et al., 1999). Powdered, then pulsed UV-irradiated, oyster mushrooms stored for 60 days at room temperature (25 ºC) or refrigerated (4 ºC) retained 62% and 71% of their vitamin $D_2$, respectively (S.-Y. Chen et al., 2015). UV-B-irradiated, air-dried, button mushrooms stored at room temperature (22 ºC) retained 54% of their vitamin $D_2$ after 12 months and 48% after 18 months (Sławińska et al., 2016).

The retention of both vitamin $D_2$ and $25(OH)D_2$ has not yet been investigated in pulse UV-irradiated whole fresh button mushrooms (*Agaricus bisporus*) that were subsequently air-dried. The aim of this study was to determine and compare the retention of vitamin $D_2$ and $25(OH)D_2$ in button mushrooms that were pulse UV-irradiated then hot-air dried and stored in similar conditions as retail dried mushrooms kept in the home.

## 2. Materials and Methods

### *2.1. Mushroom samples*

In November 2020, twelve 200 g samples (Batch 1) of fresh button mushrooms (*Agaricus bisporus*), 4.5-5.5 cm in diameter, were collected directly from a commercial farm (Costa Mushrooms) in Perth, Western Australia. A further thirteen 200 g samples (Batch 2) were collected from the same farm in January 2022. The mushrooms were harvested as they would be for market, with the stipe connected to the cap and the skirt intact. The mean weight of each mushroom was 28.6 g.

The mushrooms were transported by road in light-sealed, cooled, insulated boxes from the farm to the laboratory at Curtin University (travel time 30 min). The mushrooms were refrigerated at 4 ºC and then processed within 48 h of collection. Any residual compost was brushed from the surface of the mushrooms, and they were kept at 23 ºC for 2 h prior to processing.

*2.2. Baseline samples*

Baseline samples were prepared to allow confirmation that samples had not been inadvertently exposed to UV-radiation, such as sunlight, prior to treatment. Four 200 g fresh samples were randomly selected from Batch 1 and one 200 g sample was randomly selected from Batch 2. The samples were sliced and placed in a freezer at -20 ºC and lyophilised in a freeze dryer (Christ Alpha 1-2 LD plus, Germany) for 48 h at -30 ºC and 37 pascals. The dry matter was crushed with a mortar and pestle, and blended into a fine powder, and stored at -20 ºC.

*2.3. UV-irradiated then air-dried samples (no storage)*

Four samples of 200 g whole mushrooms from both Batch 1 and Batch 2 were UV-irradiated using a pulsed xenon lamp (Wek-tec XematicA-2L, Germany; emitting at 260-800 nm) with the skirted underside of each mushroom facing the lamp. The distance between the lamp and the mushrooms was 10 cm. The average dose of 200 mJ/cm$^2$ and a peak of 17.5 W/cm$^2$ were recorded with a radiometer (International Light Technologies ILT800 CureRight; measuring 215-350 nm). After irradiation, the mushroom samples were air-dried in a convection oven for 18 h at 60 ºC (Contherm Thermotec 2000, Contherm Scientific Ltd, New Zealand). They were frozen at -20 ºC and lyophilised for 24 h and prepared for analysis as in Section 2.2. The shorter time for freeze-drying was due to these air-dried samples having a much lower moisture content compared to the fresh samples described in Section 2.2.

*2.4. Storage for 12 months*

Four 200 g samples from Batch 1 were prepared as described in Section 2.3. Each sample was individually vacuum-sealed in plastic pouches (Laica Advanced Technology, Italy) and stored in a light-proof cardboard box at room temperature (av. 22.6 ºC, range 20 – 26 ºC; av. 50.7 % humidity, range 47-62 %) as measured by a temperature and humidity data logger (Lascar Electronics, EL-USB-2, Hong Kong) and stored for 12 months (Nov 2020-Nov 2021). Storing in vacuum-sealed pouches was to mimic the processing of vacuum-packed commercial dried mushrooms that are then stored in a pantry by the consumer without any further exposure to UV radiation (household lights emit mainly visible light and very little UV radiation). After 12 months, samples were lyophilised for 24 h and prepared for analysis as in Section 2.2.

*2.5. Storage for 3 and 6 months*

Eight 200 g samples from Batch 2 were UV-irradiated, air-dried as in Section 2.3, then stored for either 3 or 6 months under the same storage conditions as Section 2.4. During the first three months of storage the average temperature was 23 ºC (range 22 – 25 ºC) with an average humidity of 57 % (range 43-65 %). During the second three months of storage the average temperature was 21 ºC (range 18 – 25 ºC) with an average humidity of 53 % (range 40-62 %). After 3 and 6 months, samples were lyophilised for 24 h and prepared for analysis as in Section 2.2.

*2.6. Transport of samples for analysis*

After the designated storage time, samples were frozen at -20 ºC prior to being sent by overnight courier in light-sealed, insulated containers for analysis at the National Measurement Institute (NMI) in Melbourne, Australia. On arrival, they were stored at -20 ºC until analysis for vitamin $D_2$ and 25(OH)$D_2$, which occurred within 8 weeks.

*2.7. Analysis of D-vitamers*

The analysis of D vitamers was carried out using NMI's validated in-house method developed from published methodologies and approved by the National Association of Testing Authorities, Australia (ISO17025:2017). The full method and verification has been previously reported (Cardwell, Bornman, James, Daly, Strobel, et al., 2023).

In brief, freeze-dried mushroom powder (500 mg) was added to a 50 mL screw top Falcon® plastic centrifuge tube before adding 1000 mg of sodium ascorbate and 10 mL Milli-Q® water. The resultant mixture was vortexed before the addition of the internal standards of vitamin $D_2$-$^2H_3$ and 25(OH)$D_2$-$^2H_3$ (IsoSciences, Philadelphia, USA; purity $\geq$ 98%). Ethanol (30 mL) and Milli-Q® water were added to a total volume of 50 mL, followed by the addition of 2 g potassium hydroxide. The headspace of the tube was flushed with nitrogen gas to dispel oxygen and immediately capped, then placed laterally in a shaker bath at 25 ºC for 18 h for saponification. The tubes were centrifuged for 90 s at 1887*g* (Pro-Analytical, Centurion Scientific Ltd, United Kingdom). A 10 mL aliquot of the supernatant was pipetted onto diatomaceous earth extraction Chem Elut 10 mL unbuffered cartridges (Agilent Technologies, USA). After 15 minutes, 60 mL of petroleum ether (bp 40-60 ºC) were added to the extraction cartridges and the resultant eluent was evaporated with nitrogen gas in a Dionex SE500 Gas Evaporative System (Thermo Scientific, USA).

One mL *n*-heptane was added to each sample and vortexed. The final volume was transferred to glass liquid chromatography (LC) vials (1.8 mL, glass, screw-neck with 400 µL flat-bottomed glass inserts, Waters Corporation, USA) and evaporated with nitrogen before adding 800 µL of PTAD (4-phenyl-1, 2, 4-triazoline-3, 5-dione) at 2 µg/µL. The samples were allowed to form vitamin D-PTAD derivatives for 10 min, before 200 µL of Milli-Q® water were added to stop the derivatisation. Of the final volume, 400 µL were pipetted into glass LC vials. The injection volume of 10 µL was analysed by liquid chromatography coupled with triple quadrupole (LC-QQQ), tandem mass spectrometry (Agilent Technologies, 1290 Infinity Series LC System/6460 Triple Quad LC-MS fitted with a Jet Stream ESI source in positive ion mode). To ensure that only one vitamer-PTAD adduct was formed, 5 mM of methylamine was added to the mobile phase. The LC chromatographic column was a Supelco Ascentis Express C18, 15 cm x 3mm, 2.7 *µ*m (Cat #53816-U, Sigma-Aldrich, Merck, Germany). Each sample was analysed in duplicate and the average of the two results calculated. The limit of quantitation (LOQ) was 0.001 µg/g dry weight.

*2.8. Statistical analysis*

The Shapiro-Wilk test of normality was conducted on the storage times and retention of D vitamers. Wilcoxin Rank Sum tests were used to test associations between D vitamers at different storage times. Data were analysed using Stata (Version 17.0, StataCorp, USA).

Table 1. Retention of D vitamers in stored UV-irradiated, air-dried mushrooms (mean ± SD)

| | Fresh weight, g | Weight after air-drying, g | % loss [1] | Weight after freeze-drying, g | % loss [2] | $D_2$ μg/g DW | $25(OH)D_2$ μg/g DW |
|---|---|---|---|---|---|---|---|
| **Mushrooms Batch #1** | | | | | | | |
| **Pulse irradiated, air-dried, no storage** (n=4) | 201.9 ± 2.4 | 19.7 | 90.2 ± 0.2 | 17.7 | 91.2 ± 0.2 | 9.5 ± 1.4 [a] | 0.14 ± 0.03 [a] |
| **Pulse irradiated, air-dried, stored 12 months** (n=4) | 214.4 ± 6.4 | 21.3 | 90.4 ± 0.7 | 19.2 | 91.1 ± 0.4 | 5.5 ± 0.6 [a] **(57.7% retained)** | 0.10 ± 0.01 [a] **(67.9% retained)** |
| **Mushrooms Batch #2** | | | | | | | |
| **Pulse irradiated, air-dried, no storage** (n=4) | 213.3 ± 3.6 | 21.6 | 89.9 ± 0.2 | 19.8 | 90.8 ± 0.2 | 10.9 ± 0.4 | 0.16 ± 0.02 [b] |
| **Pulse irradiated, air-dried, stored 3 months** (n=4) | 219.1 ± 11.1 | 21.0 | 90.4 ± 0.7 | 19.8 | 91.0 ± 0.2 | 11.0 ± 0.8 **(100.1% retained)** | 0.14 ± 0.01 **(88.1% retained)** |
| **Pulse irradiated, air-dried, stored 6 months** (n=4) | 217.6 ± 7.2 | 21.3 | 90.2 ± 1.2 | 20.0 | 90.8 ± 0.4 | 10.1 ± 0.6 **(92.7% retained)** | 0.11 ± 0.01 [b] **(71.1% retained)** |

$25(OH)D_2$, 25-hydroxyvitamin $D_2$; DW, dry weight; LOQ, level of quantitation; SD, standard deviation
LOQ < 0.001 μg/g
[1] % weight loss in the air-dried samples compared to the fresh weight
[2] % weight loss in the freeze-dried samples compared to the fresh weight
[a] Statistically significant difference ($p < 0.05$) between 0 and 12 months of storage
[b] Statistically significant difference ($p < 0.05$) between 0 and 6 months of storage

## 3. Results

The baseline concentrations of both vitamin $D_2$ and 25(OH)$D_2$ were less than the LOQ, demonstrating that there was no inadvertent exposure to UV radiation prior to treatment.

### *3.1. Vitamin $D_2$*

Table 1 shows the concentration of vitamin $D_2$ and 25(OH)$D_2$ after 3, 6 and 12 months of storage. The average retention of vitamin $D_2$ after three months of storage was 100%, and 93% after six months, neither being statistically significant different to baseline. However, there was a statistically significant difference in vitamin $D_2$ concentration after 12 months of storage, with only 58% of the original concentration retained ($p = 0.025$), indicating that most losses of vitamin $D_2$ occurred in the second six months of storage.

### *3.2. 25-hydroxyvitamin $D_2$*

The retention of 25(OH)$D_2$ was 88% and 71% after three and six months respectively, with the latter being statistically significantly different from the baseline concentration ($p = 0.029$). After 12 months of storage there was a further drop until 68% was retained, again statistically significantly different to the original concentration ($p = 0.029$).

## 4. Discussion

This study has shown that there was little to no loss of vitamin $D_2$ in whole UV-irradiated, dried mushrooms stored for 3-6 months. Greater losses of vitamin D occurred in samples stored for 12 months and of 25(OH)$D_2$ in samples stored for 3-12 months. There have been only two other studies on the long-term retention of vitamin $D_2$ in UV-irradiated dried mushrooms. Our findings support those of a study of *A. bisporus* mushrooms, in which samples were first irradiated, then air-dried in a convection oven, before being stored in sealed bags at a room temperature of 20 ± 2 ºC (Sławińska et al., 2016). After 3, 8, 12 and 18 months, respectively, 81%, 65%, 54% and 48% of the original concentration of vitamin $D_2$ remained. The retention of vitamin $D_2$ after 12 months of storage was similar to our findings. The retention of 25(OH)$D_2$ was not measured. The second study was of oyster mushrooms that were freeze dried, powdered, then pulse UV-irradiated and kept at room temperature (25 ºC) without light exposure for 60 days, resulting in the retention of 62% of the original vitamin $D_2$ (S.-Y. Chen et al., 2015).

Two further studies have assessed vitamin $D_2$ retention in fresh UV-irradiated button mushrooms stored at room temperature over 5-8 days. One of the studies found no evidence of vitamin $D_2$

degradation in whole mushrooms after 8 days at 25 °C (Koyyalamudi et al., 2009). In sliced mushrooms stored for 5 days at 30 °C, there was no loss of vitamin $D_2$ after 4 days (Malik, Jan, Haq, et al., 2022).

We chose to irradiate fresh mushrooms before air-drying as we previously showed that this was more effective in increasing both vitamin $D_2$ and $25(OH)D_2$ concentrations, compared to irradiating dried mushrooms at the same UV dose (Cardwell, Bornman, James, Daly, Strobel, et al., 2023). Also, irradiating mushrooms after drying them does not generate any $25(OH)D_2$ (Cardwell, Bornman, James, Daly, Strobel, et al., 2023). The concentration of D vitamers would have been higher if the mushrooms were sliced or the gills were uncovered before UV exposure. Sliced mushrooms have a greater surface area than whole mushrooms and the gills have a higher concentration of ergosterol than the rest of the mushroom (Kalaras et al., 2012; Ko et al., 2008; Perera et al., 2003).

After air-drying irradiated mushrooms, the low moisture content of the dried mushrooms may slow the rate of degradation of vitamin $D_2$ (Perera et al., 2003). The half-life of $D_2$ in air-dried, UV-irradiated and powdered oyster mushrooms (*Pleurotus ostreatus*) was dependent on the storage temperature and water activity ($a_w$) in the powder (Pedrali et al., 2020). At a storage temperature of 20 ºC and a water activity of 0.32 ($a_w$ 0.32; distilled water has a $a_w$ of 1.0), the half-life of vitamin $D_2$ was 160 days, and 225 days in samples with a very low water activity ($a_w$ 0.11).

The serving size of dried mushrooms stated on commercial packs ranges between 10 and 30 g. Therefore, assuming serving size of 20 g, our data show that a serving of irradiated, then air-dried mushrooms would provide approximately 99 µg $D_2$ after 12 months storage, which is more than the recommended daily intake of vitamin D in Australia (5-15 µg in adults) (National Health and Medical Research Council, 2006), Europe (15 µg) (EFSA, 2017), the Nordic countries (10-20 µg) (Nordic Council of Ministers, 2014) and the USA (15-20 µg) (Institute of Medicine, 2011). The dose of UV radiation could be adjusted to generate D vitamer concentrations to suit local needs. UV-irradiated whole, sliced or powdered dried mushrooms can be a supplemental vitamin D food product or ingredient of functional foods, suited to those at a high risk of vitamin D deficiency (Heo, Kim, Park, & Lee, 2020). Knowing the retention of D vitamers in stored dried mushrooms is useful when recommending a specific shelf-life and storage conditions. Although the long-term effect on the retention of vitamin $D_2$ in UV-irradiated dried mushrooms, in particular button mushrooms, has not been well studied, the evidence from the current study suggests that sufficient

vitamin $D_2$ is present after 12 months of storage in darkness at 20-25 ºC to be of nutritional significance.

A major strength of this study was the use of a sensitive and specific analytical method that has been validated for a mushroom matrix. There was a chain of custody from the farm to the laboratory so there was no inadvertent exposure to UV radiation. This was confirmed by analysing a fresh sample at the laboratory to ensure the concentrations of D-vitamers were below the LOQ. Although two different batches of mushrooms were analysed, they originated from the same farm under the same growing conditions, were irradiated and air-dried with the same equipment and the same method and were analysed using the same method. The D vitamer concentrations measured prior to storage were similar in both batches.

## 5. Conclusions

Mushrooms are dried to increase the shelf life and make them easier to transport and store without refrigeration. UV-irradiated dried mushrooms are a good source of D vitamers. Vitamin $D_2$ and 25(OH)$D_2$ concentrations show a gradual loss over 12 months, with the greatest rate of loss occurring during the latter 6 months. However, vacuum-sealed, UV-irradiated dried mushrooms kept at room temperature have a high retention of D-vitamers over 6 months, a time span in which it is expected that most dried mushrooms would be cooked and consumed. Even after 12 months storage, UV-irradiated dried mushrooms still offered a valuable nutritional source of vitamin $D_2$ in a 20 g serving size.

**Author Contributions:** G.C.: Conceptualization, investigation, writing – original draft preparation; J.F.B.: Supervision, review and editing; A.P.J.: Supervision, review and editing; A.D.: Formal analysis; E.D.: Review and editing; G.D.: Methodology; P.A.: Resources; L.J.B.: Supervision, review and editing. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The data presented in this study are available upon request from the corresponding author. The data are not publicly available.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Chapter 5: Effect of household cooking on the retention of vitamin $D_2$ and 25-hydroxyvitamin $D_2$ in pulse UV-irradiated, air-dried button mushrooms (*Agaricus bisporus*)**

**Objective 4:** To analyse the retention of D vitamers in UV-irradiated, dried mushrooms after being rehydrated and then cooked by three different common methods.

The study had determined that D vitamers were more efficiently generated by irradiating mushrooms prior to drying and that there is good retention of those vitamers even after 12 months of storage. At the time of conducting this study there were only two studies on the retention of vitamin $D_2$ in cooked mushrooms, one on fried fresh wild mushrooms (P. Mattila et al., 1999), and the other fried, baked and boiled fresh button mushrooms (Ložnjak & Jakobsen, 2018). There were none on the retention in cooked dried mushrooms.

Results of a pilot study were reported at the 4th Collaborative Research Symposium of the National Measurement Institute:
Cardwell G., Bornman J.F., James A.P., Strobel N., Black L.J. (2019). **An exploratory study on the retention of vitamin $D_2$ in bio-fortified dried button mushrooms after household cooking**. 4th Collaborative Research Symposium, Victoria University, Melbourne, 22nd November 2019

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# Effect of household cooking on the retention of vitamin $D_2$ and 25-hydroxyvitamin $D_2$ in pulse UV-irradiated, air-dried button mushrooms (*Agaricus bisporus*)

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## Abstract

Mushrooms generate vitamin $D_2$ when exposed to ultraviolet (UV) radiation from solar radiation or a UV lamp. The aim of the study was to determine the true retention of vitamin $D_2$ and 25-hydroxyvitamin $D_2$ (25(OH)$D_2$) after cooking UV-irradiated, air-dried, then rehydrated button mushrooms (*Agaricus bisporus*). Mushrooms were exposed to pulsed UV radiation, then air-dried in a convection oven, followed by rehydration in warm deionised water. Samples were cooked in three different ways: frying (5 min), baking (10 min, 200 ºC) and boiling (20 min, 90 ºC). After cooking, there was a high retention of D vitamers (≥95%), with frying and baking resulting in significantly higher retention compared to boiling ($p$<0.0001). UV-irradiated, dried mushrooms are a valuable source of vitamin $D_2$ after rehydration and cooking by common household methods.

**Keywords**: 25-hydroxyvitamin $D_2$; *Agaricus bisporus*; air-drying; cooking; pulsed ultraviolet radiation; true retention; vitamin $D_2$

## 1. Introduction

There is a high global prevalence of vitamin D deficiency (Amrein et al., 2020; K. D. Cashman, 2022) and low vitamin D intakes have been widely reported (Ahmed et al., 2021; Dunlop et al., 2023; Herrick et al., 2019; Lips et al., 2019). Few foods are a good source of vitamin D, and these are mainly limited to foods of animal origin, such as fish, meat, egg yolk, along with fortified foods. Mushrooms are commonly consumed and are a potential non-animal source of vitamin D (Cardwell et al., 2018). Although mushrooms are usually treated as a vegetable from a culinary perspective, they are not a plant and reside in their own biological kingdom, namely fungi. It is well established that when mushrooms are exposed to a source of ultraviolet (UV) radiation, such as solar radiation or a UV lamp, they convert ergosterol to pre-vitamin $D_2$, which is then thermally

isomerised to vitamin $D_2$ in high concentrations in a temperature-dependent process (Jasinghe et al., 2007; Keegan et al., 2013).

There is usually no vitamin $D_2$ in commercially cultivated mushrooms as they receive only (non-UV) fluorescent light during picking and packing; however, a 100 g serve of fresh UV-exposed mushrooms can produce 20-160 $\mu$g of vitamin $D_2$ depending on duration and intensity of UV radiation (Kalaras et al., 2012; Koyyalamudi et al., 2011; Kristensen et al., 2012; Urbain & Jakobsen, 2015). Therefore, this popular vegetarian food has the potential to counteract vitamin D deficiency and help people to meet the recommended daily dietary intake of 5-20 $\mu$g of vitamin D, depending on regional recommendations (EFSA, 2017; Institute of Medicine, 2011; National Health and Medical Research Council, 2006; Nordic Council of Ministers, 2014)

Air-drying mushrooms is a simple method used to inhibit spoilage from microorganisms, permit storage at room temperature, and extend the shelf life, which are important attributes for populations with little or no access to refrigeration. Dried mushrooms are commonly used in Asian cuisine, yet they have not been well studied as a source of vitamin D. A survey of 35 species of dried mushrooms available in China revealed that all samples contained vitamin $D_2$ (average 16.9 $\mu$g/g dried mushroom; range 7.7-25.0 $\mu$g/g), most likely generated by sun exposure during the drying process or during growth of the wild varieties (Huang et al., 2016).

The impact of cooking on the retention of vitamin $D_2$ has been explored in fresh UV-exposed mushrooms (Ložnjak & Jakobsen, 2018; Malik, Jan, Haq, et al., 2022; P. Mattila et al., 1999; Salemi et al., 2021). To our knowledge, there are no studies of vitamin $D_2$ retention in dried mushrooms that were subsequently cooked. Hence, the aim of this study was to investigate the effect of common household cooking methods on the retention of both vitamin $D_2$ and 25-hydroxyvitamin $D_2$ (25(OH)$D_2$) in UV-irradiated, air-dried, common button mushrooms (*Agaricus bisporus*), one of the most highly consumed mushroom species in the world (Royse et al., 2017).

## 2. Materials and methods

### *2.1. Mushroom samples*

Forty-one 200 g samples of fresh button mushrooms, 4.5-5.5 cm in diameter, were collected directly from a farm (Costa Mushrooms) in Perth, Western Australia, and transported by road to Curtin University in light-sealed, cooled, insulated boxes (travel time 30 min). The mushrooms were harvested as for market, comprising cap, stipe and the gills still skirted. The mean weight of

each mushroom was 24.2 g (standard deviation (SD) 1.0, range 23.0-25.7 g). Samples were refrigerated at 4 ºC and prepared within 48 h of collection. Any residual compost was brushed from the mushrooms, which were kept at room temperature (23 ºC) for 2 h prior to further processing.

The fresh mushroom samples were irradiated, followed by air-drying, then randomly allocated to one of five groups for the following processes:

1) Rehydrated, not cooked (control);
2) Rehydrated, dry-fried 5 min;
3) Rehydrated, baked 10 min;
4) Rehydrated, boiled 20 min in deionised water at pH 5.5;
5) Rehydrated, boiled 20 min in deionised water at pH 3.5.

In addition, one random, non-irradiated fresh sample was analysed for D vitamers to confirm there had been no exposure to UV radiation at the farm or during transportation.

*2.2. Preparation of rehydrated control samples*

Each sample (8 x 200 g) was UV-irradiated under a pulsed xenon lamp (Wek-tec XeMaticA-2L, Germany; emitting at 260-800 nm) with the skirted gills facing the lamp. The average dose was 200 $mJ/cm^2$, with an average peak of 17.5 $W/cm^2$, based on five pulses at 7 volts as measured with a radiometer (International Light Technologies ILT800 CureRight; bandwidth measured 215-350 nm). The distance from the surface of the mushrooms to the lamp was 10 cm. Each mushroom was then placed in a single layer on shelves and air-dried in a convection oven for 18 h at 60 ºC (Contherm Thermotec 2000, Contherm Scientific Ltd, New Zealand).

The samples were rehydrated in deionised water for 2 h in a covered stainless-steel saucepan. The initial water temperature for that and all subsequent rehydrated samples was 50 ºC, reducing to 36 ºC after 60 min, and 28 ºC at 120 min (Micron Q1090 multimeter, Micron Technology, USA). The internal mushroom temperature during rehydration was the same as the water temperature. Excess water was removed via sieve and paper towelette before the samples were weighed, sliced and placed in a freezer at -20 ºC overnight then lyophilised (Christ Alpha 1-2 LD plus, Germany) at -30 ºC and 37 pascals for 48 h, crushed with a mortar and pestle, then blended to a fine powder.

*2.3. Preparation of dry-fried mushroom samples*

Each 200 g sample (n=8) was irradiated and air-dried as per Section 2.2, then vacuum-sealed and stored at 4 ºC for four days, before being rehydrated and excess water removed, in the same

manner as per Section 2.2, prior to frying. A stainless-steel frying pan was pre-heated for 10 min on a gas stove. Within 60 min of rehydration, each 200 g sample was dry-fried, independently of each other, for 5 min. The internal mushroom temperature was measured randomly via the stipe directly after completion of frying with a multimeter probe (n=23; mean internal temperature 53 ºC; standard deviation (SD) 5; range 43-63 ºC; median 53 ºC). Each sample was then weighed, sliced and placed in a freezer at -20 ºC overnight then lyophilised as described in Section 2.2.

*2.4. Preparation of baked mushroom samples*

Each 200 g sample (n=8) was irradiated, air-dried, vacuum-sealed and stored at 4 ºC for three days until rehydration and preparation for cooking as in Section 2.2. Samples were baked for 10 min in a pre-heated fan-forced LG oven (Model LF66105SS) at 200 ºC (Avanti Tempwiz oven thermometer). The mean internal mushroom temperature directly after 10 min of baking was 56 ºC (n=12; SD 8; range 43-65 ºC; median 57 ºC). After baking, the samples were weighed, sliced and placed in a freezer at -20 ºC, then lyophilised as per Section 2.2.

*2.5. Preparation of boiled mushroom samples*

Each 200 g sample (n=16) was irradiated, air-dried, vacuum-sealed and stored at 4 ºC for four days until rehydration for 2 h in deionised water as in Section 2.2. Samples were boiled for 20 min in 500 mL deionised water at 90 ºC in a stainless-steel saucepan within an hour of rehydration. The water temperature was checked every 5 min to ensure the temperature remained constant. The samples were boiled separately at either of two pH levels. Samples (8 x 200 g) were boiled in deionised water that had a native pH of 5.3-5.6 (mean 5.5), while another 8 x 200 g samples were boiled in deionised water with a pH of 3.5 (TPS AQUA-pH meter, Queensland, Australia), adjusted by the addition of fresh lemon juice. After 20 min boiling, the internal temperature (n=14; mean 82 ºC, SD 4; range 74-88 ºC; median 82 ºC)was measured randomly in individual mushrooms. The samples were then drained with a sieve and dried with a paper towelette, weighed, sliced and placed in a freezer at -20 ºC, then lyophilised as per Section 2.2.

*2.6. Dry weight (DW) determination*

Prior to analysis, all samples were freeze-dried as per Section 2.2 until there was no further weight loss (48 h). The samples were weighed using an electronic compact balance (EK-4100i, A&D Technology, USA).

*2.7. Rehydration ratio*

As described in Section 2.2, the dried mushrooms were rehydrated in 50 ºC deionised water for 2 h. They were weighed directly before rehydration, and after 60 and 120 min of rehydration, with the rehydration ratio (RR) determined by the equation:

RR = Rehydrated weight (g) / Air-dried weight (g)

*2.8. Weight loss and true retention*

The samples were weighed initially in their fresh state, then after each step of air-drying, rehydrating, each cooking procedure, and after freeze-drying (Table 1). To determine the percentage weight loss attributed to cooking, compared to the rehydrated weight, we used equation 1 below and to determine the true retention of D vitamers we used equation 2, which we adapted from Murphy and colleagues (Murphy, Criner, & Gray, 1975).

Equation 1:

$$\%\ \text{weight loss} = \left(\frac{weight\ of\ rehydrated\ sample\ before\ cooking\ (g) - weight\ of\ sample\ after\ cooking\ (g)}{weight\ of\ rehydrated\ sample\ before\ cooking\ (g)}\right) * 100$$

Equation 2:

$$\%\ True\ retention = \left(\frac{Cooked\ sample{:}\ [(D\ vitamer\ \mu g/g\ DW\ x\ total\ DW\ (g)) \div FW\ (g)]}{Control\ sample{:}[D\ vitamer\ \mu g/g\ DW\ x\ total\ DW\ (g) \div \text{FW (g)}]}\right) * 100$$

DW, dry weight; FW, fresh weight

*2.9. Transport of samples for analysis*

Lyophilised samples were stored at -20 ºC for two weeks before being sent for analysis at the National Measurement Institute (NMI) in Melbourne, Victoria, Australia. Sealed, freeze-dried samples were placed in an insulated, light-sealed container with frozen cool bricks for overnight transport to the NMI. On arrival, samples were kept frozen at -20 ºC before measurement of vitamin $D_2$ and 25(OH)$D_2$ within 8 weeks.

*2.10. Analysis of D vitamers*

Vitamin $D_2$ and 25(OH)$D_2$ were measured using a validated in-house method at NMI (ISO17025:2017) approved by the National Association of Testing Authorities, Australia, and previously described (Cardwell, Bornman, James, Daly, Strobel, et al., 2023). It was developed from published methodologies (Jäpelt et al., 2013; Jones & Makin, 2000; Strobel et al., 2013). The entire analysis was conducted in a laboratory protected from UV radiation.

Freeze-dried mushroom powder (500 mg) was added to a 50 mL screw top Falcon® plastic centrifuge tube before the addition of 1000 mg of sodium ascorbate and 10 mL Milli-Q® water. The resultant mixture was vortexed before the addition of the internal standard of vitamin $D_2$-$^2H_3$ and 25(OH)$D_2$-$^2H_3$ (IsoSciences, Philadelphia, USA; purity ≥ 98%). Ethanol (30 mL) and Milli-Q® water were added to a total volume of 50 mL. The tubes were then capped and hand-shaken, followed by the addition of 2.0 g potassium hydroxide. The remaining headspace of the Falcon® tube was flushed with nitrogen gas to minimise potential oxidation during saponification. The tube was immediately capped and placed laterally in a shaker bath for saponification at 25 ºC for 18 h.

The tubes were centrifuged for 90 s at 1887 *g* (~ 3750 rpm, radius 12 cm: Pro-Analytical, Centurion Scientific Ltd, United Kingdom). A 10 mL aliquot of the supernatant was pipetted onto diatomaceous earth extraction Chem Elut 10 mL unbuffered cartridges (Agilent Technologies, USA). After 15 minutes, 60 mL of petroleum ether (boiling point 40-60 ºC) were added to the extraction cartridges and the resultant eluent was evaporated with nitrogen gas in a Dionex SE500 Gas Evaporative System (Thermo Scientific, USA).

One mL *n*-heptane was added to each sample and vortexed. The final volume was transferred to 1.8 mL glass liquid chromatography (LC) vials (Waters Corporation, USA) and evaporated with nitrogen before adding 800 $\mu$L of 4-phenyl-1, 2, 4-triazoline-3, 5-dione (PTAD) at 2 $\mu$g/$\mu$L. The samples were allowed to form vitamin D-PTAD derivatives for 10 min, before 200 $\mu$L of milli-Q water were added to stop the derivatisation. Of the final volume, 400 $\mu$L were pipetted into LC vials. The injection volume of 10 $\mu$L was analysed by LC-QQQ (Agilent Technologies, 1290 Infinity Series LC System/6460 Triple Quad LC-MS fitted with a Jet Stream ESI source in positive ion mode). To ensure that only one vitamer-PTAD adduct was formed, 5 mM of methylamine was added to the mobile phase. The LC chromatographic column was a Supelco Ascentis Express C18, 15 cm x 3mm, 2.7 $\mu$m (Cat #53816-U, Sigma-Aldrich, Merck, Germany). Each sample was analysed in duplicate and the average of the two results calculated. The limit of quantitation (LOQ) was 0.001 $\mu$g/g DW for both vitamin $D_2$ and 25(OH)$D_2$.

*2.11. Statistical analysis*

Wilcoxin rank sum tests were used to test the differences in freeze-dried weight between the control sample and the combined cooked samples, and the differences in vitamin $D_2$ concentrations between the control sample and the combined cooked samples. For the data used in the calculation of the retention of D vitamers, a natural log transform was used, and the results were back-transformed for reporting. The mean fresh weight of the control mushrooms was used

as the denominator in the retention formula (see Section 2.8, equation 2). A one-way analysis of variance (ANOVA) with Tukey's pairwise comparisons was used to test statistically significant differences in the retention of vitamin $D_2$ and 25(OH)$D_2$ between cooking methods. ANOVA was also used to test the differences in freeze-dried weight between the control mushrooms and the cooked samples. Data were analysed using Stata (Version 17.0, StataCorp, USA).

## 3. Results and discussion

### *3.1. D vitamers in fresh mushrooms*

The concentration of vitamin $D_2$ and 25(OH)$D_2$ in fresh mushrooms on arrival at the laboratory was below the Level of Quantitation (LOQ), confirming that there was no exposure to UV radiation at the farm or during transportation.

### *3.2. Air drying of fresh mushrooms*

A temperature of 60 ºC was chosen as it is the common air-drying temperature used in commercial settings (Argyropoulos et al., 2011; Giri & Prasad, 2009). Samples were irradiated before air-drying as that has been found to be more efficient at generating D vitamers than air-drying prior to irradiation (Cardwell, Bornman, James, Daly, Strobel, et al., 2023). The air-drying method was similar to that used in four studies of dried mushroom: three with sliced white button mushrooms and one with whole shiitake mushrooms (Doymaz, 2014; García-Segovia, Andrés-Bello, & Martínez-Monzó, 2011; Giri & Prasad, 2007, 2009).

### *3.3. Rehydration of dried mushrooms*

During the rehydration process the water temperature dropped from an initial 50 ºC to a mean of 36 ºC after 1 h, and 28 ºC after 2 h. The water temperature was allowed to decline over time in order to replicate preparation in a domestic kitchen where dried mushrooms are left to soak in warm water for one or two hours before cooking. The samples approximately doubled their weight after 1 h of rehydrating in warm water and were 2.2 times their weight after 2 h. Between 90-94% of the total rehydration over 2 h was achieved in the first hour. The swift initial water uptake was possibly due to the rapid filling of cavities between cells as the absorption rate begins to decline once the hyphae and intercellular spaces fill with water (García-Segovia et al., 2011).

Table 1. Mushroom sample weight (mean ± SD) after each treatment process

| Treatment | Fresh weight (g) | Air-dried weight (g) | % weight loss [1] | Rehydrated weight (g) | RR | Cooked weight (g) | % weight loss [2] | Freeze-dried weight (g) | % weight loss [3] |
|---|---|---|---|---|---|---|---|---|---|
| Fresh [4] | 199.2 | N/A | N/A | N/A | N/A | N/A | N/A | 19.2 | 90.4 |
| Irradiated, dried, rehydrated (control) [5] | 209.1 ± 5.1 | 25.4 ± 4.0 | 88 ± 2 | 57.0 ± 4.9 | 2.2 | N/A | N/A | 12.0 ± 0.9 | 94.2 ± 0.4 [b] |
| Irradiated, dried, rehydrated, dry-fried [5] | 207.0 ± 5.3 | 23.9 ± 2.4 | 89 ± 1 | 47.7 ± 4.8 | 2.0 | 35.3 ± 1.5 | 26.0 ± 3.1 | 10.9 ± 0.3 | 94.7 ± 0.2 [a,b] |
| Irradiated, dried, rehydrated, baked, oven [5] | 206.9 ± 5.8 | 22.3 ± 1.9 | 89 ± 1 | 48.6 ± 2.7 | 2.2 | 31.9 ± 2.3 | 34.4 ± 3.8 | 11.3 ± 1.4 | 94.5 ± 0.5 [a,b] |
| Irradiated, dried, rehydrated, dried, boiled, pH 5.5 [5] | 206.8 ± 5.9 | 23.4 ± 1.6 | 89 ± 1 | 52.8 ± 3.3 | 2.3 | 51.0 ± 2.9 | 3.4 ± 5.1 | 9.6 ± 0.7 | 95.4 ± 0.3 [a,b] |
| Irradiated, dried, rehydrated, dried, boiled, pH 3.5 [5] | 205.6 ± 4.2 | 21.9 ± 1.7 | 89 ± 1 | 51.5 ± 3.7 | 2.4 | 49.9 ± 3.2 | 3.1 ± 5.5 | 8.9 ± 0.5 | 95.7 ± 0.3 [a,b] |

N/A, not applicable; SD, standard deviation; RR, rehydration ratio

[1] sample weight loss after air-drying compared to fresh weight

[2] sample weight loss after cooking compared to rehydrated weight

[3] sample weight loss after freeze-drying compared to fresh weight

[4] 1 x 200 g independent sample

[5] 8 x 200 g independent samples

[a] Statistically significant difference ($p < 0.05$) between the boiled samples and the dry-fried or baked samples

[b] Statistically significant difference ($p < 0.05$) between the control samples and the cooked samples

*3.4. Weight differences in freeze-dried samples*

Compared with the control, the cooked samples had a higher loss of solid matter after freeze-drying ($p$ <0.05). Boiling the rehydrated mushrooms resulted in a statistically significantly higher loss of solid matter, and hence a lower freeze-dried weight, compared to any of the other cooking methods ($p < 0.0001$ in all comparisons). Compared to fresh mushrooms, the process of rehydrating and cooking may have caused a loss of cellular matter, possibly via dissolving and leaching of dried mushroom particles into the surrounding water.

*3.5. D vitamer retention in cooked, rehydrated, UV-irradiated, air-dried mushrooms*

The true retention of vitamin $D_2$ was 109 % for dry-fried mushrooms, 108 % for baked, 100 % for mushrooms boiled at pH 5.5, and 101 % for those boiled at pH 3.5. The true retention was statistically significantly lower for vitamin $D_2$ ($p < 0.0001$) when mushrooms were boiled (at either pH 5.5 or 3.5) compared to mushrooms that were dry-fried or baked. The difference in retention rate of vitamin $D_2$ between mushrooms that were baked or dry-fried was not statistically significant. There was no significant difference in the retention rate of $D_2$ between boiling the samples in water of either pH 3.5 or pH 5.5.

The true retention of 25(OH)$D_2$ was 100 % for dry-fried mushrooms, 97 % for baked, 95 % when boiled at pH 5.5, and 96 % for those boiled at pH 3.5. Comparisons between the retention rates for 25(OH)$D_2$ were not statistically significantly different.

It may not be valid to compare the retention of D vitamers in the current study of cooked, rehydrated dried mushrooms to studies of cooked fresh mushrooms. The true retention of D vitamers during cooking of dried mushrooms may be different to that of fresh mushrooms due to the effect of drying on the cellular structure. However, it appears that the true retention of vitamin $D_2$ in this study of cooked dried mushrooms is generally higher than that found in fresh cooked button mushrooms, which has been reported as 94% (roasted) and 92% (boiled) (Salemi et al., 2021), 88% (boiled), 75% (roasted) and 67% (fried in oil) (Malik, Jan, Haq, et al., 2022), 81-88% (fried, no oil), 62-67% (baked), and 62-80% (boiled) (Ložnjak & Jakobsen, 2018).

Table 2. True retention of D vitamers in dried, rehydrated and cooked mushrooms (mean ± SD)

| | Cook temp (ºC) | Time (min) | Mushroom internal temp (ºC) | Vitamin $D_2$ $\mu g/g$ DW | True retention % vitamin $D_2$ | 25(OH)$D_2$ $\mu g/g$ DW | True retention % 25(OH)$D_2$ |
|---|---|---|---|---|---|---|---|
| Fresh [2] | N/A | N/A | N/A | <LOQ [1] | N/A | <LOQ [1] | N/A |
| Irradiated, dried, rehydrated (control) [3] | N/A | N/A | N/A | 14.8 ± 3.0 [a] | N/A | 0.25 ± 0.03 | N/A |
| Irradiated, dried, rehydrated, dry-fried [3] | n.m. | 5 | 53 ± 5.0 | 17.9 ± 1.9 [a] | 109 ± 3 [b] | 0.27 ± 0.03 | 101 ± 3 [c] |
| Irradiated, dried, rehydrated, baked, oven [3] | 200 ºC | 10 | 56 ± 7.5 | 17.7 ± 3.1 [a] | 108 ± 4 [b] | 0.23 ± 0.05 | 97 ± 4 |
| Irradiated, rehydrated, dried, boiled, pH 5.5 [3] | 90 ºC | 20 | 82 ± 3.9 | 16.6 ± 1.0 [a] | 100 ± 2 [b] | 0.25 ± 0.04 | 95 ± 4 [c] |
| Irradiated, rehydrated, dried, boiled, pH 3.5 [3] | 90 ºC | 20 | 82 ± 3.9 | 17.9 ± 1.6 [a] | 101 ± 2 [b] | 0.28 ± 0.07 | 96 ± 6 |

25(OH)$D_2$, 25-hydroxyvitamin $D_2$; DW, dry weight; LOQ; level of quantitation; N/A, not applicable; n.m., not measured; SD, standard deviation

[1] LOQ <0.001 $\mu g/g$

[2] 1 x 200 g independent sample

[3] 8 x 200 g independent samples

[a] $p < 0.05$ difference between the control samples and the cooked samples

[b] $p < 0.0001$ difference between the boiled samples and the dry-fried and baked samples

[c] $p = 0.05$ between the dry-fried sample and the sample boiled at pH 5.5

### *3.5.1. Boiling*

There was nearly complete retention of D vitamers in boiled, rehydrated dried mushrooms at the two different pH values compared to the control samples (Table 2). There was no statistically significant difference in D vitamer retention between rehydrated dried mushrooms boiled at either pH 3.5 or 5.5. There was an expectation that there would be a difference in D vitamer retention at the two different pH values based on the results of another study (Ložnjak & Jakobsen, 2018). In that study, the retention of vitamin $D_2$ was 80% when whole fresh mushrooms were boiled for 20 min in water with added lemon juice to a pH of 3.5, compared to 62% when boiled in neutral-pH water. The authors of that study suggested that the lemon juice preserved the vitamin D during cooking, possibly due to the antioxidant properties of ascorbic acid (Hajimahmoodi et al., 2012). Two further studies of fresh UV-irradiated button mushrooms that were boiled (the water pH was not documented) also found a high vitamin $D_2$ retention of 92 % (Salemi et al., 2021) and 88 % (Malik, Jan, Haq, et al., 2022).

### *3.5.2. Frying*

Dry-fried mushrooms retained all their D vitamers compared to the control samples (Table 2). This was a higher level of retention than found in two studies of fresh mushrooms that were also fried without added fat for 5 min. In that study, the true retention was 81% in whole fried button mushrooms (Ložnjak & Jakobsen, 2018), and 80-82% and 97-100% in fried wild mushrooms (*Cantharellus cibarius* and *Cantharellus tubaeformis*, respectively) (P. Mattila et al., 1999). In comparison, another study showed that frying fresh sliced UV-irradiated button mushrooms in oil resulted in a 67% retention of vitamin $D_2$, possibly due to the fat-soluble vitamin $D_2$ leaching into the oil (Malik, Jan, Haq, et al., 2022).

### *3.5.3. Baking*

The baked mushrooms retained 108 % of their vitamin $D_2$ and 97 % of $25(OH)D_2$, which is higher than in other studies of baked or roasted fresh UV-irradiated mushrooms. In a similar study, but using whole fresh button mushrooms, there was 67% retention of vitamin $D_2$ in mushrooms baked under the same conditions as the current study (Ložnjak & Jakobsen, 2018). Two other studies of vitamin $D_2$ retention in roasted fresh UV-irradiated button mushrooms found 75% retention in sliced fresh mushrooms (Malik, Jan, Haq, et al., 2022), and a 94% retention in whole fresh mushrooms (Salemi et al., 2021).

There is a consistency in that all studies of cooked fresh and dried UV-irradiated mushrooms have shown a high retention of vitamin $D_2$ such that they offer nutritionally relevant amounts of D vitamers to the consumer. Dried wild mushrooms and sun-dried commercial mushrooms may also have the potential to be a source of D vitamers when cooked (Huang et al., 2016; Rangel-Castro et al., 2002). Commercial packs of dried mushrooms usually state a serving size as 10-30 g. Using an example serving size of 20 g, the UV-irradiated, air-dried then rehydrated control samples would provide approximately 140 $\mu$g $D_2$, which is higher than the recommended daily requirements for vitamin D. The UV dose could be modified to generate amounts of vitamin D to suit the local requirement for dietary vitamin D, or the dried mushrooms could be ground into a powder to provide a supplemental source of vitamin D for use in dishes such as soups and stews, or as an ingredient in manufactured food products (Heo et al., 2020). The European Union has approved the sale of fresh UV-exposed mushrooms provided that the tolerable upper intake limits of vitamin D set by the European Food Safety Authority (EFSA) are not exceeded (European Commission, 2018). In 2019, EFSA also approved the use of UV-exposed mushroom powder as a novel food ingredient (Turck et al., 2020).

## 4. Strengths and limitations

The mushrooms were collected directly from the farm and analysis of the fresh mushrooms showed that the D vitamers were below the limit of quantitation, confirming they had not been inadvertently exposed to sunlight or other source of UV radiation prior to treatment. The irradiation dose was measured between each sample treatment to provide an average dose over the entire exposure process and to ensure that irradiation was consistent over time. Although the current study used common drying and rehydration times and temperatures, the D vitamer retention may vary depending on drying and rehydrating conditions. The rehydration ratio may have been higher if rehydration was maintained at a constant water temperature and continued until there was no further weight gain. However, the rehydration method was designed to mimic home rehydration prior to cooking. Only one dose level of UV radiation was used in this study since it has been previously reported that the dose was sufficient to provide a nutritionally useful concentration of D vitamers (Cardwell, Bornman, James, Strobel, & Black, 2019); different doses will generate different concentrations of D vitamers.

## 5. Conclusion

In conclusion, UV-irradiated, air-dried mushrooms that were rehydrated in warm water and then cooked, retained a high proportion of their D vitamer content. Dried mushrooms that have been biofortified with D vitamers via UV radiation can be a very useful, convenient and affordable source of vitamin $D_2$ even after cooking using common household methods. They are likely to benefit populations without access to refrigeration, people avoiding animal derived products for religious or cultural reasons, and those at a high risk to vitamin D deficiency.

## CRediT authorship contribution statement

**Glenn Cardwell:** Conceptualisation, Investigation, Methodology, Data curation, Writing – original draft. **Janet F. Bornman:** Methodology, Writing – review & editing. **Anthony P. James:** Methodology, Writing – review & editing. **Alison Daly:** Formal analysis, Writing – review & editing. **Georgios Dabos:** Analysis, Methodology**. Paul Adorno:** Resources**. Jette Jakobsen:** Methodology, Writing – review & editing. **Eleanor Dunlop:** Writing – review & editing. **Lucinda J. Black**: Methodology, Writing – review & editing.

## Data availability

Data will be made available on request.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Funding sources had no involvement in the study design, data collection, analysis and interpretation of data, in the writing of the article, or in the decision to submit the article for publication.

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**Abbreviations**

$25(OH)D_2$: 25-hydroxyvitamin $D_2$; DW: dry weight; FW: fresh weight; LOQ: limit of quantitation; LC-QQQ: Liquid chromatography with triple quadrupole tandem mass spectrometry; UV: ultraviolet

**Chemical compounds studied in this article**

Vitamin $D_2$/ergocalciferol (PubChem CID: 5280793); 25-hydroxyvitamin $D_2$/25-hydroxyergocalciferol (PubChem CID: 5710148).

## Chapter 6: Discussion

The thesis has met its objectives and satisfied the aim of determining the efficacy of producing dried mushrooms that have been bio-fortified with D vitamers through UV irradiation, and of documenting their retention and nutrient value to the consumer after common household storage and cooking conditions.

### *6.1. Generation of D vitamers in dried mushrooms*

The published review in the journal *Nutrients* showed that there was very little research on the generation and retention of D vitamers in dried mushrooms, despite their popularity in Asian cuisine. The only study of commercially available dried mushrooms revealed that vitamin $D_2$ was detected in all the 35 mushroom species collected on the Chinese market (Huang et al., 2016). They had an average vitamin $D_2$ content of 17 $\mu$g/g, therefore a 20 g serving would provide about 340 $\mu$g, which is well above the daily requirement. The author stated the presence of vitamin $D_2$ was due to the mushrooms being either grown outside or sun-dried. It is surprising that the Chinese producers have not exploited this characteristic when exporting to other countries, including Australia, where vitamin D deficiency is so prevalent. Other producers of dried mushrooms, such as France, Poland and South Korea could also offer vitamin D-enhanced varieties, labelling them as such and establishing them as a regular a source of vitamin D, especially as we now know they retain much of the D vitamers during storage.

In European countries, North America and Australia, where labour costs would prevent sun-drying of mushrooms from being economically viable, it makes sense to have an automated system where mushrooms pass under UV-emitting lamps with a set dose such that an accurate vitamin D claim can be made on the label. The most efficient method would be to use a pulsed UV lamp at a dose to provide at least 20 $\mu$g $D_2$/serve with less than a second of exposure.

In 2018 the European Food Safety Authority (EFSA) approved ‘UV-treated mushrooms’ as a novel food with a limit of 20 $\mu$g $D_2$/100g fresh weight, although it was not clear if dried mushrooms are also approved under that legislation (European Commission, 2018). On the other hand, in 2019, EFSA also approved vitamin $D_2$ mushroom powder as a novel food (Turck et al., 2020), therefore one can only presume that UV-irradiated dried mushrooms

would also be permitted to be marketed in the European Union, offering a very useful means to boost the vitamin D content of foods.

*6.2 Ideal sequence of UV irradiation and drying mushrooms*

The study described in Chapter 3 made it clear it was much more effective to irradiate mushrooms prior to drying, rather than after, although the latter sequence still produced nutritionally relevant concentrations of D vitamers. None of the major mushroom growing companies in Australia produce dried mushrooms; they produce only fresh button mushrooms. To my knowledge, all dried mushrooms sold in Australia are imported. Should a company choose to provide dried mushrooms then it makes sense to design their farm such that fresh mushrooms are irradiated straight after harvesting, then placed in an air-dryer before being vacuum sealed and labelled with their average D vitamer content. That sequence would be linked to cost saving, such as using less electricity and lamp life being extended There is a future where all mushrooms are pulse UV-irradiated, such that fresh, dried and powdered mushrooms can be a very useful source of D vitamers.

*6.3. Retention of D vitamers in pulse UV-irradiated stored dried mushrooms*

This study detailed in Chapter 4 found that there is a gradual loss of D vitamers over 12 months in home storage conditions, with most of the loss being in the second six months. Sufficient D vitamers remained even after 12 months to provide at least the daily requirement in a 20 g serving of dried mushrooms. It is not clear what are the degradation products of D vitamers. A study of the degradation of vitamin $D_3$ under different conditions found that it can transform back to pre-vitamin $D_3$ and isomers of vitamin $D_3$ (Mahmoodani, Perera, Fedrizzi, Abernethy, & Chen, 2017). Another paper commented that, as those degradation reactions for vitamin $D_3$ did not involve the side chain (C22 onwards), then the degradation reactions would likely be the same for vitamin $D_2$ (Pedrali et al., 2020).

Foods with a shelf life less than two years must provide a ‘best-before’ date according to Australian Food Standard 1.2.5. Producers of UV-irradiated dried mushrooms are likely to stamp their product with a ‘best-before’ date of 12 months after packaging. There could be 6 to 12 months between packaging the mushrooms, transporting (e.g. exporting via sea), storing at the retailer, and storing at home before being used. The protein, fat, carbohydrate, fibre and sodium content are unlikely to change over 12 months, but the food regulators will

want assurance that a nutrient claim still holds throughout the 12 months of potential shelf life.

*6.4. Retention of D vitamers in pulse UV-irradiated dried mushrooms after cooking*

A food manufacturer making a nutrient claim need only show that the nutrient was present in the stated amounts at the time of production and packaging. The nutrient claim does not relate to the nutrient content at the 'use by' or 'best before' date (Australian New Zealand Food Standard Code 1.2.5). Some nutrients such as vitamin C and folate are heat labile, so cooking is likely to dramatically reduce their concentration. For example, fresh green peas have 32 mg vitamin C per 100 g, but when boiled that level drops to 14 mg/100 g (Australian Food Composition Database). Although UV-irradiated dried mushrooms are an excellent source of D vitamers it would be disingenuous to make a vitamin D claim if rehydrating and cooking made them a poor source of D vitamers. Hence the very important question: How much D vitamer is retained in UV-irradiated dried mushrooms after rehydration in warm water, followed by common household cooking methods? The answer was that virtually all the D vitamers were retained, making UV-irradiated dried mushrooms a convenient source of vitamin D. The Food Standards Code (1.2.7-11) states: "*A nutrition content claim must be stated together with a statement about the form of the food to which the claim relates*", therefore the D vitamer claim could only be made for cooked dried mushrooms as consumed.

*6.5. Bioavailability of vitamin $D_2$ from mushrooms and the potential benefits to bone health*

Although it was not an aim of the thesis, it is important to know if the vitamin D produced in UV-exposed mushrooms can be absorbed from the digestive tract and have a physiological function. In the case of vitamin D, it has to first be released or extracted from the food matrix and then arrive at the brush border of the intestinal wall so that it can be absorbed. There are many factors that influence the bioavailability of vitamin D in food, such as the food matrix that encapsulates the vitamin D, the type of vitamin D, composition of the diet (e.g. fat enhances the absorption of vitamin D), age, genetics and the vitamin D status of the consumer (Borel, Caillaud, & Cano, 2015).

A range of human studies have attested to the bioavailability of vitamin $D_2$ from mushrooms. A systematic review paper of six randomised control trials reported that the largest effect of UV-exposed mushrooms on total serum 25(OH)D was when participants

had a low vitamin D status (<50 nmol/L in blood serum) (K. D. Cashman, Kiely, Seamans, & Urbain, 2016). There was little effect when the vitamin D status was high as the rise the in serum 25(OH)$D_2$ was accompanied by a reduction in serum 25(OH)$D_3$. Since that publication, a further study of 28 adults found that mushroom-derived vitamin $D_2$ consumed over 12 weeks caused a rise in serum 25(OH)$D_2$ and a drop in serum 25(OH)$D_3$ without significantly changing the total 25(OH)D concentrations (Pinto et al., 2020).

In addition, there is evidence that vitamin $D_2$ biofortified mushrooms contribute to bone health in animal models. A study of UV-B-irradiated, freeze-dried, powdered shiitake mushrooms given to rats showed a significant increase in the mineral density of the femur bone compared to that of the control rats on a vitamin D-deficient diet (Jasinghe et al., 2005). There was also an increase in the mineral density of the femur bone in rats fed UV-B exposed button mushrooms (Calvo et al., 2013; Malik, Jan, Al-Keridis, et al., 2022) and UV-B exposed shiitake mushrooms (Won, 2019). Pulsed irradiated oyster mushrooms and UV-B irradiated shiitake mushrooms also improved femur bone density in osteoporotic mice (S. Y. Chen et al., 2015; G. Lee et al., 2009).

Although the evidence for improved bone health with mushroom-derived vitamin $D_2$ is in animal models, oral vitamin $D_2$ supplements were used for a long time to prevent rickets in children and osteomalacia in adults (Rajakumar, Greenspan, Thomas, & Holick, 2007), so it seems likely that mushroom-derived vitamin $D_2$ (and 25(OH)$D_2$) can be a useful adjunct to the diet and may benefit human bones, especially in those with a low vitamin D status.

### *6.6. Nutritional benefits of mushrooms*

As mushrooms are neither plant nor animal, they have been described as the “Third Food Kingdom” (Feeney et al., 2014). Mushrooms are a convenient and nutritional food, widely available in supermarkets in the western world. Mushrooms offer a lot more than just D vitamers. Button mushrooms are a particularly good source of other vitamins, such as riboflavin ($B_2$), niacin ($B_3$), pantothenic acid and biotin, and minerals, including selenium and copper (Feeney et al., 2014; Roupas, Keogh, Noakes, Margetts, & Taylor, 2012).

Adding a serve of mushrooms (in this case 84 g) to the average American diet added valuable micronutrients while not having any impact on the energy, sodium or fat intake (Fulgoni & Agarwal, 2021). Their analysis found that fibre increased by 5-6%, copper by

24-32%, potassium, riboflavin, niacin and selenium by about 13%, and choline by 5-6% in both adolescents and adults. If the mushrooms had been UV-irradiated to a level of 5 µg vitamin D per serving, then vitamin D intake would have doubled.

### *6.7. Strengths and limitations*

The author maintained the chain of custody throughout the collection, storage, treatment and transport of each sample. All samples in the studies came from the same mushroom farm and were grown in the same substrate. All treatments in the studies used the same pulsed UV-irradiation machine, radiometer, air-dryer and freeze-dryer. The mushrooms in each study were all the same mean diameter, and the same stage of maturity, and were irradiated with the same orientation (gill side towards the lamp). Commercial mushrooms are very homogeneous in nature, so there is little expected variation between samples. It is possible to compare data among each of the studies, since each sample received the same dose of UV radiation and were air-dried at the same temperature and for the same duration.

The methodology for D vitamer extraction and analysis was validated and approved by NATA in May 2017, and then was verified for this thesis in a mushroom matrix in April 2019. The analyses were all conducted in the same laboratory, with the same equipment at the National Measurement Institute.

Ergosterol, or pro-vitamin $D_2$, was not measured. However, ergosterol is not a limiting factor in the generation of D vitamers in mushrooms as there is about 1000 times more ergosterol than vitamin $D_2$ in the mushrooms used in this series of experiments (Gallotti & Lavelli, 2020; Hammann et al., 2016).

Analysing more samples would have been preferable and would have provided more treatment options in each study; however, one constraint on the number of samples was the high cost of analysing D vitamers due to the price of the internal standards and the two-day labour-intensive process of extraction, followed by overnight liquid chromatography coupled with triple quadrupole mass spectrometry. At the time of writing, each sample cost $610AUD (approx. $US420) to analyse for D vitamers. There were no other analysis options hence the laboratory used the only validated and verified method of extraction of D vitamers from a mushroom matrix. With advances in methodology, the cost of analysis may

be reduced which should encourage further research on the D vitamer concentration in all foods, and specifically in the full range of edible mushrooms.

The research was conducted solely on button mushrooms (*Agaricus bisporus*). Further similar research on commonly consumed dried mushrooms, such as oyster mushrooms, is warranted to determine if there are similar observations regarding the retention of D vitamers during storage and cooking. It would also be helpful to conduct a small sensory study to see if the UV treated dried mushrooms had similar organoleptic properties to untreated dried mushrooms when cooked. If there is little difference then mushroom companies can take vitamin D-enhanced dried mushrooms to market with confidence.

### *6.8. UV radiation and the marketing of vitamin D-enriched dried mushrooms*

Radiation is a term that can have negative connotations for the public who associate the word 'radiation' with radioactivity, disease and mutation. However, there are two types of radiation:

a) Ionising radiation, which breaks chemical bonds resulting in the release of electrons. This is an effective method of reducing or eliminating microorganisms to improve the safety and extend the shelf life of foods. If food is treated with ionising radiation (e.g. gamma rays) as a form of pest control, then they must be labelled as such according to Food Standards Australia New Zealand (https://www.foodstandards.gov.au/consumer/foodtech/irradiation/Pages/default.aspx).

b) Non-ionising radiation, such as the UV radiation used in this thesis. Non-ionising radiation does not need to be mentioned on a food label as it poses no threat to human health.

In Australia, there is no requirement to mention the exposure to UV radiation when packaging vitamin D-enhanced mushrooms. Should consumers ask how the vitamin D content is increased the growers can mention that mushrooms are placed under a UV lamp for a short time with a similar effect to exposing them to sun light.

## Chapter 7: Future directions and conclusions

### *7.1. Future directions and recommendations*

There are very few studies on the effect of UV irradiation on the nutrient levels of mushrooms (aside from D vitamers). Deliberate exposure to UV-B radiation did not reduce the B vitamins in button mushrooms, although exposure to sunlight caused a statistically significant loss of riboflavin (vitamin B2) and provided evidence of folate loss (Simon, Borzelleca, DeLuca, & Weaver, 2013). Sun-drying may also have reduced the riboflavin content of shiitake mushrooms (Huang et al., 2016). It is expected that there would be little effect on the macronutrients protein, fat and carbohydrate, and the minerals such as selenium, but there appears to be potential for UV radiation to affect vitamins, such as the B group vitamins. Nevertheless, UV irradiation of mushrooms increases their $\beta$-glucan content and their antioxidant activity (Tiwari et al., 2021). $\beta$-glucans have the potential to help control blood glucose and blood lipid levels, as well as having immunomodulatory and anti-inflammatory properties (Cerletti, Esposito, & Iacoviello, 2021).

This thesis used pulsed UV radiation, exposing the mushrooms to five pulses with each pulse being approximately 100 $\mu$seconds i.e. a total duration of about half a second. One could speculate that at that level of exposure there would be minimal effect on the vitamin level, excluding vitamin $D_2$. On the other hand, commercial UV lamps require up to 30 minutes exposure to generate D vitamers, and one could speculate there would be a drop in vitamin content under those conditions. A study of the nutrient content of UV-exposed mushrooms, comparing pulsed UV vs conventional lamp, would be a worthwhile study. This thesis has given insight into the effect of cooking on the retention of D vitamers in dried button mushrooms. However, it needs to be reproduced in other common globally consumed mushrooms.

Any future vitamin D claim will also need to take into account the degradation of D vitamers over time, hence it would be wise to know the D vitamer retention in cooked irradiated dried mushrooms after 12 months of storage. There is one published study (Sławińska et al., 2016), and the submitted study in Chapter 5, regarding the retention of D vitamers in stored dried mushrooms. However, there is no study on D vitamer retention after cooking dried mushrooms that have been stored for 6 or 12 months. The D vitamer retention

in such mushrooms is still likely to be high, but it would be useful to test that conjecture to support any vitamin D claim.

If there was negligible nutrient loss after pulsed UV irradiation then mushroom producers could be convinced to design their farms to expose all mushrooms, both fresh and dried, to UV radiation so they can make a vitamin D claim on all labels. Irradiating fresh mushrooms and either packing them immediately or drying them for later vacuum sealing in plastic pouches, would ideally suit the producers. Fresh mushrooms are sold either loose or in punnets in Australia. Mushrooms packed in punnets at the farm reassure the consumer that no-one has touched their mushrooms. Being sold in punnets also allows a Nutrition Information Panel to be placed on the plastic wrapping where a nutrient claim can be included.

More mature mushrooms have their gills exposed, and the gills have the highest concentration of ergosterol, compared to the cap or stipe. Applying a higher dose of UV radiation to the gills would generate more D vitamers, meaning that fewer mushrooms (fresh, dried or powdered) would be required to give the daily requirements of vitamin D. This would be an easy option for farmers. It would mean, in particular, that less powdered mushroom would be required as an ingredient in functional foods.

It is difficult to obtain data about the worldwide dried mushroom market, however one marketing website estimates that the world dried mushroom market is 14 billion USD (https://www.futuremarketinsights.com/reports/dried-mushroom-market), while another suggests it is only 4 billion USD (https://www.alliedmarketresearch.com/dried-mushroom-market-A31627), underlining our lack of credible information. Countries that produce dried mushrooms (imported mainly from China, South Korea, France and Poland) have the opportunity to expose their mushrooms to either a UV lamp or the sun after harvesting and during the drying process. These mushrooms could be marketed as "vitamin D" mushrooms and make a nutrient claim on their label, depending on the importing country.

Possibly, the most viable option with vitamin D-enhanced dried mushrooms is as a powdered condiment sprinkled on meat or vegetarian dishes, or an ingredient in soups and stews, or designed as an ingredient for functional foods. The European Food Safety Authority Panel on Nutrition, Novel Foods and Food Allergens has concluded that UV-

irradiated mushroom powder with 1000-1300 $\mu$g $D_2$/g is safe to use in novel foods and beverages for the general population (Turck et al., 2020). The Panel concluded that mushroom powder is safe as a food ingredient when consumed in amounts that provide up to 15 µg vitamin $D_2$ per day to people 12 months or older. The Panel also noted that it was unlikely for anyone to be consuming amounts >15 µg vitamin $D_2$ daily. This gives mushrooms farmers a potential market to offer bio-fortified mushroom powder to food manufacturers, commercial kitchens and food supplement producers. The powder can be made into a useful condiment that provides both umami (a savoury flavour) and vitamin D, meaning it could be added to soups, stews, pies etc. to boost flavour and vitamin D.

A study of UV-irradiated, air-dried oyster mushrooms ground into a powder concluded that it could be a viable functional food ingredient (Pedrali et al., 2020). The study observed the half-life of vitamin $D_2$ under different water activity and temperature (20-40 ºC) conditions. The half-life varied from 57-225 days and followed first-order kinetics, with 225 days being achieved at the lowest moisture content and stored at 30 ºC. The authors state that their study can be used as a basis to design a formulation strategy for the use of mushroom powder in functional foods.

The European Union has also approved the sale of fresh UV-exposed mushrooms as a novel food as long as the label makes it clear that the mushrooms have been treated with UV radiation (European Commission, 2018). Example expressions to be used on labels include: "controlled light treatment was used to increase vitamin D levels" or "UV treatment was used to increase vitamin $D_2$ levels". Any country permitting the sale of vitamin D-enhanced fresh, dried or powdered mushroom will need to consider the potential of a high long-term consumption causing toxicity, although this risk will likely be much lower than the over-consumption of vitamin D supplements.

### *7.2. Conclusions*

Button mushrooms are the most consumed mushrooms in Australia and New Zealand and one of the most consumed mushrooms in the world (Royse, 2014). This study has shown that UV-irradiated, air-dried button mushrooms not only generate high concentrations of D vitamers, those D vitamers are well retained during storage and cooking. At each stage of this thesis, realistic parameters were given to simulate the conditions most likely to occur during processing (UV-irradiation and air-drying, vacuum packing) and in the home

(common cooking methods and storage). Hence the results are directly applicable to the producer and the consumer.

Although this project focussed on UV-irradiated dried whole mushrooms, they can easily be furthered commercially processed into a mushroom powder, which also has the potential to be a useful source of vitamin $D_2$, as an ingredient in home cooking or added to processed foods during manufacture.

The advantage of both dried and powdered mushrooms as a potential source of vitamin D is that they are easy to transport, affordable, convenient, have a long shelf life, and no refrigeration is required. Vitamin D deficiency is common throughout the world. Approximately 1 in 4 Australian adults are vitamin D-deficient, being particularly prevalent in those that are dark skinned, cover their skin for cultural or religious reasons, or remain indoors during daylight hours. Vitamin D-enhanced, dried mushrooms have the potential to prevent or reverse vitamin D deficiency in Australia and many parts of the world.

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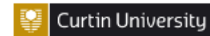
## Appendix I: Conference posters

Cardwell G., Black L.J., Bornman J.F, James A.P., Strobel N. Pulsed UV radiation increases the vitamin D concentration of fresh and dried mushrooms in a dose dependent manner. 5th International Vitamin Conference, 8-10 August 2018, Sydney, New South Wales

National Measurement Institute




### PULSED UV RADIATION INCREASES THE VITAMIN D CONCENTRATION OF FRESH AND DRIED MUSHROOMS IN A DOSE DEPENDENT MANNER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**Introduction**

Fresh commercially grown mushrooms generally have low concentrations of vitamin D. However, they generate nutritionally significant concentrations of ergocalciferol (vitamin D2) and 22-dihydroergocalciferol (vitamin D4) when exposed to a source of ultraviolet (UV) radiation, such as sunlight or a UV lamp. Mushrooms deliberately exposed to UV radiation post-harvest can easily attain a total of 10 µg vitamin D/100g fresh weight[1,2], equivalent to the recommended daily intake in many countries. We investigated the effect of pulsed UV radiation on the generation of D vitamers in Australian fresh and dried button mushrooms (*Agaricus bisporus*).

**Methods**

Fresh white and brown button mushrooms were harvested from the same commercial mushroom farm. Fresh mushrooms were then subjected to either 2, 4 or 8 pulses of UV radiation (Xenon Corporation RC-847 pulsed UV lamp), giving 505J/pulse or *ca* 1.1 J/cm² per pulse, then freeze-dried and analysed by triple quadrupole mass spectrometry for vitamins $D_2$, $D_3$, and $D_4$, and the hydroxylated forms of vitamins $D_2$ and $D_3$ (25(OH) $D_2$ and 25(OH)$D_3$ respectively) at the National Measurement Institute, Melbourne, Australia. A second mushroom sample from the same harvest was air-dried for 20h at 60°C before being exposed to pulsed UV radiation and analysis for D vitamers. The drying process reduced the moisture content from 92% to 8%, a similar moisture content to that found in commercial dried mushrooms.

**Vitamin D content of the common white and brown button mushroom (*A. bisporus*) in µg/100g fresh weight**

| Vitamer | White fresh (2 pulses) | White fresh (4 pulses) | White fresh (8 pulses) | White dried (2 pulses) | White dried (4 pulses) | White dried (8 pulses) | Brown fresh (2 pulses) | Brown fresh (4 pulses) | Brown fresh (8 pulses) | Brown dried (2 pulses) | Brown dried (4 pulses) | Brown dried (8 pulses) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D2 | 16 | 50 | 76 | 39 | 51 | 76 | 17 | 64 | 80 | 45 | 65 | 74 |
| 25(OH)D2 | 2.7 | 5.7 | 8.4 | 0.0 | 0.0 | 0.0 | 1.0 | 3.6 | 3.6 | 0.0 | 0.0 | 0.0 |
| D4 | 0.5 | 1.4 | 2.6 | 1.2 | 2.0 | 3.1 | 0.4 | 1.4 | 1.5 | 1.0 | 1.8 | 2.3 |

**Results**

There was less than 1.0 µg/100g fresh weight of all forms of vitamin D in the control (non-irradiated) samples. Concentrations of vitamin $D_3$ and 25(OH)$D_3$ in UV-irradiated mushrooms were less than the level of detection. As anticipated, pulsed UV-radiation increased the vitamin $D_2$ concentrations in brown and white button mushrooms, with little difference in the vitamin D concentration between the two strains of button mushrooms (Table 1). Vitamin $D_4$ also increased in fresh and dried mushrooms after UV-irradiation. There was no detectable 25(OH)$D_2$ in dried brown and white mushrooms. Dried mushrooms had a greater capacity to generate vitamin $D_2$ compared to fresh mushrooms after 2 pulses, but there were no discernible differences after 4 and 8 pulses.

**Conclusion**

The preliminary results show that UV-irradiation of both fresh and dried mushrooms is effective in raising the vitamin $D_2$ and $D_4$ concentrations. To our knowledge, this is the first study to show that UV-irradiation of mushrooms increased the 25(OH)$D_2$ in fresh mushrooms but not in dried mushrooms. It is conceivable that UV radiation post-harvest (for fresh mushrooms) or post-drying (for dried mushrooms) could become standard commercial practice. Such a practice has the potential to make mushrooms an important dietary source of vitamin D and thus help address the vitamin D deficiency in the Australian populace, where one in four adults have vitamin D deficiency[3].

Brown and white button mushrooms after 8 pulses (about 3 seconds) of UV-radiation. No visible difference to non-irradiated mushrooms.

Xenon pulsed UV-radiation machine

HPLC- Triple Quadrupole Mass Spectrometer


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# D vitamers in pulse UV-irradiated air-dried button (*A. bisporus*) mushrooms


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## Introduction

The few sources of dietary vitamin D are mainly animal products such as fish, meat and egg yolk. Fresh and dried mushrooms naturally generate vitamin $D_2$ when exposed to a source of ultraviolet (UV) radiation, such as sunlight or a UV lamp [1]. They can be a valuable source of vitamin D for those at high risk of vitamin D deficiency.

## Aim

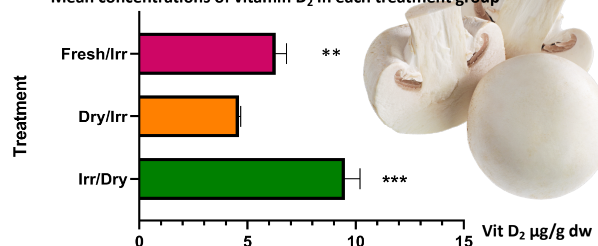
To determine if there is a difference in the concentrations of vitamin $D_2$ and 25-hydroxyvitamin $D_2$ (25(OH)$D_2$) in dried button mushrooms (*Agaricus bisporus*) when pulsed UV-irradiated either before or after air-drying.

## Method

Fresh button mushrooms (16 x 200 g) were irradiated with a total of 200 $mJ/cm^2$ pulsed UV radiation before or after being air-dried at 60 °C for 18 h. Fresh mushrooms were also irradiated at the same dose, with no subsequent air-drying. Control mushrooms were fresh and untreated. The D vitamers were quantified in freeze-dried samples using liquid chromatography triple quadrupole mass spectrometry at the National Measurement Institute. We did ANOVA and pairwise comparisons with a Tukey correction test for differences in vitamin $D_2$ between treatments and a t-test for the difference in 25(OH)$D_2$ values.

## Results

Mushrooms UV-irradiated before air-drying had double the concentration of vitamin $D_2$ than those irradiated after drying (9.46 µg/g dw vs 4.60 µg/g dw). In comparison, irradiated fresh mushrooms that did not undergo drying provided 6.31 µg/g dw. The concentration of 25(OH)$D_2$ in dried mushrooms irradiated before drying was 0.14 µg/g dw. There was no detectable 25(OH)$D_2$ in the untreated controls, nor in the mushrooms irradiated after drying; however, fresh irradiated mushrooms had 0.05 µg/g dw.




Mean vitamin $D_2$ concentration (µg/g dry weight [dw];) bars show standard error of the mean (SEM). Fresh/Irr, fresh UV-irradiated; Dry/Irr, dried, then UV-irradiated; Irr/Dry, UV-irradiated, then dried
** p = 0.003 compared to UV-irradiated, then dried; *** $p < 0.0001$ compared to dried, then UV-irradiated

## Conclusions

To our knowledge, this is the first study to assess the effect of pulsed UV radiation on the generation of vitamin $D_2$ and 25(OH)$D_2$ in dried button mushrooms. Other studies used conventional UV lamps and analysed only vitamin $D_2$ [2,3]. At the dose we used, exposing mushrooms to UV radiation before drying generated more vitamin $D_2$ and 25(OH)$D_2$ than irradiating after drying. Commercially dried, UV-enhanced mushrooms can be a valuable, long shelf life, source of vitamin D.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**Appendix II: Additional data to the paper in Chapter 5.**

**Effect of household cooking on the retention of vitamin $D_2$ and 25-hydroxyvitamin $D_2$ in pulse UV-irradiated, air-dried button mushroom (*Agaricus bisporus*)**

The following was not an aim of the study, but it did produce two interesting observations.

**D vitamer concentration of UV-irradiated dried mushrooms**

Mushrooms taken directly from the farm had no D vitamers (all being < LOQ), which was expected. The mean D vitamer content of the irradiated dried mushrooms was 10.9 $\mu$g ± 0.37 for vitamin $D_2$ and 0.16 $\mu$g ± 0.02 for 25(OH)$D_2$, which was similar to that found previously in the first study (Chapter 3) being 9.5 $\mu$g ± 1.4 and 0.14 $\mu$g ± 0.03, respectively. This was to be expected as they received the same treatment. The D vitamer values appear to increase after rehydration and again after cooking. When the dried mushrooms were rehydrated, the vitamin $D_2$ rose to 14.8 µg/g DW (Table 2, p 66). While not statistically significant, the vitamin $D_2$ concentrations in the rehydrated mushrooms were higher than those found in the dried mushrooms (median = 10.9 versus median = 15.4, z = 1.96, $p$ = 0.06). There was a statistically significant difference in D vitamer concentration between rehydrated dried mushrooms and the cooked samples (see Table 2, Chapter 5). It is possible that when UV-irradiated dried mushrooms rehydrate in warm water there is further temperature-dependent conversion of pre-vitamin $D_2$ to vitamin $D_2$.

**Weight differences in freeze-dried samples**

There was a difference in the freeze-dried weight between the irradiated, dried mushrooms and in those that were subsequently rehydrated and cooked (median 19.6 g *vs* 12.1 g, z = 2.72, $p$ = 0.004; Table 1, p 64). After being freeze-dried to remove all moisture, it was revealed that fresh mushrooms were 9.6% solids, i.e. fresh mushrooms were 90.4% moisture, a level of moisture commonly reported in fresh button mushrooms. Air-dried mushrooms that were rehydrated prior to freeze-drying showed a lower content of dry solids (5.8%). Rehydrated, then cooked mushrooms, also showed less dry solids than fresh mushrooms, with dry-fried 5.3%, baked 5.5%, boiled (pH 5.5) 4.6 %, and boiled (pH 3.5) 4.3% solids. Fresh mushrooms had a statistically significantly higher percentage of solid

matter compared with air-dried, rehydrated, cooked mushrooms ($p < 0.001$ in all comparisons). The process of rehydrating dried mushrooms therefore caused some loss of cellular matter into the rehydrating medium. I do not know of another study showing the loss of the dry mushroom matrix after rehydration.

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### Paper 1: A review of mushrooms as a potential source of dietary vitamin D
### Paper 3: Vitamin $D_2$ and 25-hydroxyvitamin $D_2$ retention in pulse UV-irradiated dried button mushrooms (*Agaricus bisporus*) after 3, 6 and 12 months of storage

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