Project Title:	Understanding Mushroom Nutrition: Project aimed at improving yield, substrate efficiency and utilisation and flavour
Project Number:	M 56
Project Leader:	Dr Kerry Burton East Malling Research (EMR)
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Date project completed:	30 September 2015

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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Dr Kerry Burton

Senior Research Leader

East Malling Research

Signature ..... Date .....

Report authorised by:

Dr Mark Else

Programme Leader, Research Efficiency & Crop Production

East Malling Research

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GROWER SUMMARY

Headline

- Mushroom yields increased 11.5% by addition of protein-based supplements at 1.5% (ProMycel Gold, Champfood E, MCSubstradd and Natural Gold (a lipid-protein blend) to phase 3 composts
- The increased mushroom yield is worth 6.4 times the cost of supplement (at 1.5%)
- Protein-based supplements increased mushroom cap density and so improved texture and picking rates
- Supplementation did not affect mushroom flavour.

Background

The nutrition from compost is a key factor for the successful mushroom growth. A range of different nutritional supplements are available to the mushroom industry, some reported to correct possible nutrient deficiencies in compost and others reported to have 'hormonal' effects by stimulating extra growth. At the start of this project there was little objective knowledge on the effects of these supplements in phase 3 compost on mushroom yield, quality and metabolism. This project evaluated the effects of four different types of compost supplement (protein-based, lipid-protein blend, carboxylic acid-based and mineral micronutrients) applied to two different phase 3 composts (horse manure-based and straw-based). Mushroom yield was measured as weight of mushrooms harvested. Mushroom quality was measured as mushroom colour, density (related to texture), percentage dry weight and mushroom flavour. Examining the effects of supplements on flavour is a novel and important component of the project as flavour is key to consumer acceptance, so it is important to know whether there are positive or negative effects from supplements.

Because different supplements have reported to have different modes of action (correcting deficiencies of different nutrients or hormonal) this project also examined the biology of the *A. bisporus* mycelia growing in different nutrient conditions to develop our understanding on mushroom nutrition. This work involved measuring gene expression using microarray technology.

The four supplement types under evaluation are available as commercial products:

- Protein-based (largely from soy) – three products tested (ProMycel Gold, Champfood E, MCSubstradd)
- A blend of lipid and protein-based product (Natural Gold)
- A carboxylic acid-based product (MycroNutrient)
- Mineral micronutrients, a mix of calcium, magnesium, sulphur, boron, copper, iron, manganese, molybdenum and zinc salts (Micromax)

Summary

This project examined supplementation of phase 3 compost: a review of current usage, crop experimentation to identify possible benefits to yield and quality, and laboratory analysis to examine how supplements may affect the physiology of the mycelium.

Compost supplementation is practiced throughout Europe. There are a range of product types (protein, lipid/protein blend, carboxylic acids and mineral micronutrients) and different suppliers. However, there is little objective knowledge on how effective these products are; most information is provided by the suppliers. The normal rate of use is 1.5% (for protein-based supplements) on white strains but there is reduced use of supplements for brown strains.

The four protein-based supplements (ProMycel Gold, Champfood E, MCSubstradd and Natural Gold) produced an average increase in mushroom yield of 11.5% on white strain A15. No differences were found between these supplements. This equates to a 6.4 times multiplier in terms of cost benefit [£1 spent on supplement produces £6.40 of extra mushrooms]. No increase in yield was detected by supplementation with carboxylic acids and mineral micronutrients. The carboxylic acids product (MycroNutrient) is marketed as a ‘Casing Supplement’; it was included in these experiments for completeness. Previous reports from USA have suggested that composts may be deficient in mineral micronutrients, the evidence from these experiments suggest that the composts used (produced in Britain and The Netherlands) are not mineral deficient.

The effects of supplements on a range of mushroom quality attributes (mushroom colour, texture (density), flavour and dry matter content) were investigated. Protein-based

supplements improved mushroom density by 5.5%, which directly relates to mushroom texture and also picking rates (more fresh weight picked per mushroom of the same size). The use of supplements produced only minor or no differences in the other quality attributes. Two protein supplements (ProMycel Gold and McSubstradd) did make the mushrooms more yellow coloured, this is probably below the threshold of consumer detection and the overall whiteness (L value) was unaffected. However, the small effects of supplements on mushroom yellowness were affected by compost type and flush number and it is therefore difficult to be sure of an overall trend.

One protein-based supplement was found to increase the yield of the brown strain, Heirloom. This is of interest to growers of 'browns' as they are considered by some to be unresponsive to supplements.

The compost type was found to influence mushroom quality but not yield: horse-manure-based composts produced more dense mushrooms while straw-based compost produced whiter mushrooms.

Gene expression analysis was carried out on the mycelium growing in the differently supplemented composts to try to identify how the extra yields are achieved. The composts of the first flush showed that only one gene had different expression levels in the protein supplemented compost compared with the non-supplemented compost. This was a surprise and may be because the mycelium has sufficient capacity in terms of enzymes produced to deal with the extra protein provided. A larger number of genes had changed expression levels in the lipid/protein treatment compared with the non-supplemented treatment. The up-regulated genes were largely concerned with nitrogen metabolism while many of the down-regulated genes had lipid metabolism functions. This is evidence that the lipid/protein supplement does change the metabolism of the mycelium. However, the yield increase of the lipid/protein supplement was the same as the protein alone supplement.

Financial Benefits

The financial benefits of supplementing phase 3 compost with protein supplement (at 1.5%) to improve yields have been calculated as: that for every £1 spent on protein supplement £6.4 of extra mushrooms are produced.

For this calculation Brendan Burns (of Sylvan Inc) kindly provided figures for realistic approximate costs of supplements and compost and wholesale price of mushrooms.

- Price of protein supplement £400/tonne
- Price of Phase 3 compost £145/tonne
- Price of mushrooms (ex-farm i.e. harvested and packed) £1.40 to £1.50/kg
- Net Price of mushrooms (excluding harvesting and packing) £0.90/kg

From Experiments 2 and 3:

Mushroom yield	(No supplementation)	363 kg/tonne compost
Mushroom yield	(Average of the 4 protein supplements)	405.8 kg/tonne compost
Increase in yield due to protein supplements (405.8 - 363)		42.825 kg/tonne compost

Value of supplementation (increased mushroom yield X net price of mushrooms)

i.e. (42.825 X £0.90) **£38.54**

Cost of supplement (at 1.5% rate i.e. 15 kg/tonne compost or 0.015tonne of supplement)

(0.015 tonne supplement X £400/tonne) **£6**

£6 spent of supplement yields an extra £38.54 of mushrooms

Therefore, the multiplier of supplement use is £38.54/£6 = 6.4 times

An additional cost of using supplements may be the additional cooling requirement (electricity cost), particular during the summer.

Action Points

- Protein-based supplements offer such clear benefits without negative issues (other than potential over-heating of compost) that their use should be regularly reviewed. In our experiments the four protein-based supplements gave similar increases to yield and we were unable to distinguish between them. They are of course different products and the choice between them may be more than price but also the degree of temperature spike, possible benefits specific to individual farms/composts and the other components present in the supplement. For instance, Natural Gold is a lipid/protein blend and other supplements contain mineral trace elements, rape (lipids) and polysaccharides

- There is the possibility that the supplement inclusion rate of 1.5% could be slightly raised for further increases in yield if the danger of overheating compost can be controlled
- The gene expression laboratory experiment as part of this project gives good grounds to believe that supplementation with lipids offers future possibilities of further yield improvements. Growers are recommended to ‘keep a watching eye’ on this
- The one negative attribute of protein-based supplement was the slight increased yellowing of mushrooms. It is not believed that this is a problem although growers are recommended that they again ‘keep a watching eye’ in case yellowing should ever become a significant quality issue
- Growers of brown strains are recommended to trial protein-based supplements

SCIENCE SECTION

Introduction

Growth and development of most living organisms are highly dependent on nutrition. This maxim of biology applies to saprotrophic fungi such as the cultivated mushroom, *Agaricus bisporus*, which derives its nutrition from partially degraded leaves. Cereal straw is composted by a two-step process (before the introduction of *A. bisporus* inocula) which changes the structure, chemistry and biology of the substrate so that it is suitable for *A. bisporus* mycelial growth. During composting the straw is wetted, opened up both from macroscopic to molecular scales, the structural polymer, lignocellulose, is partially degraded and micro-organisms grow sequestering many nutrients such as nitrogen, phosphorus and sulphur. Some of the by-products of lignin breakdown are humic substances which enable favourable growth of *A. bisporus* mycelium compared to any competing fungi.

However, compost that is selective for the growth of *A. bisporus* mycelium is not necessarily optimal for the growth of *A. bisporus* mushrooms. A number of nutritional supplements products (referred to hereafter as 'supplements') are available for the mushroom industry. Research in the 1980s demonstrated significant increases in mushroom yield by the addition of protein-based supplements, such as soy products, to phase 2 composts. Since that time, the industry has largely changed to phase 3 composts and a wider range of supplements have been made available to the industry. These include:

- Protein-based supplements from soy and biological waste sources
- Lipid-based supplements thought to act in a hormonal manner by changing the physiology of *A. bisporus* mycelium
- Carboxylic acid-based products (these are largely marketed as casing supplements)
- Mineral micronutrients (originally derived for crop plant agriculture) to correct mineral deficiencies which may exist in the compost. Micromax contains mix of calcium, magnesium, sulphur, boron, copper, iron, manganese, molybdenum and zinc salts

There has been little impartial research on the effectiveness of these products on phase 3 composts, most of the information coming from the supplement producers or held as proprietary information by growers and composters. The broad aims of this project are to re-evaluate the use of nutritional supplements in the light of the range of product-types

available and phase 3 compost. A review was conducted on the range and usage of supplements in Europe. Experiments were designed and carried out to identify the effects of supplements on mushroom yield and mushroom quality In addition, research was conducted to investigate how supplements might change mushroom physiology to improve yield and quality. Whole genome microarrays (enabled by the successful international Agaricus Genome Project – completed 2012) were employed to examine gene expression of *A. bisporus* genes in composts with different supplement contents.

Materials and Methods

The review of nutritional supplement usage

The review of nutritional supplement usage was conducted by talking to growers in the UK, Ireland, The Netherlands, Belgium and Poland, at the Mushroom Days exhibition (May 2013), HDC Mushroom Panel meetings, visits to farms and during conversations at the MushTV project meetings. We also conducted specific consultations with supplement suppliers (Table 1, below).

Table 1: Supplement suppliers and contact names

Supplement suppliers	Contacts
Amycel	John Kidder, John Clay, Hubert Hay
Champfood	Eric de Nooij
Lambert	Scott McIntyre
MCSubstradd Havens	Bart de Leeuw
NutriGain	Stuart Whitehall, Frank Parker

The mushroom growth trials

Three mushroom cropping trials were conducted at Moreton Pinkney Mushroom Farm, Moreton Pinkney, Northamptonshire, UK. For all three trials, three replicate trays were used for each supplement x compost type, and six replicate trays for un-supplemented controls. The experimental layout involved 'blocking' to improve the statistical analyses: one tray of each supplemented treatment or two trays of un-supplemented controls were arranged in three areas (blocks) in the growing room.

Trial One (Oct/Nov 2013) came about due to a delivery error as the phase 3 compost had been spawned with the brown strain Heirloom. However, the researchers decided to utilise

the opportunity and investigate the effects of supplements on Heirloom. This straw-based compost was supplemented as indicated in Table 2 and compared with non-supplemented phase 3 compost. For comparison, two trays of A15 growing on a horse manure-based phase 3 compost were also grown and the yields determined. These A15 data are shown in Figure 2 (in Results section) but have not been subject to statistical analysis due to the low replication.

Table 2: Supplements and their rate of addition used in Trial 1 (Oct/Nov 2013)

Supplement Type	Product Name	Rate of Supplementation (w/w)
Protein	Champfood E *	1.5%
Protein	MCSubstradd Soya	1.5%
Protein	ProMycel Gold *	1.5%
Lipid/protein blend	Natural Gold	1.5%
Carboxylic acid-based Δ	MycroNutrient	0.5%
Carboxylic acid-based Δ	MycroNutrient	0.25%
Mineral-based nutrient ‡	Micromax	0.15%
Mineral-based nutrient ‡	Micromax	0.23%

* At the time of writing, the manufacturer's respective web-sites describe ProMycel Gold as "*A balanced nutritional content of lipids, carbohydrates, micronutrients, and multiple protein sources*" and Champfood to contain *vitamins, minerals and trace elements*"

Δ Micronutrient is recommended by manufacturers as a Casing Supplement; it was applied here to the compost to be comparable with other treatments

‡ Micromax contains mix of calcium, magnesium, sulphur, boron, copper, iron, manganese, molybdenum and zinc salts.

Trial Two (Jan/Feb 2014): Two phase 3 compost types were used: a straw-based compost and a horse manure-based compost. The spawn used for both composts was A15 (Sylvan Inc.). The phase 3 composts were filled into growing trays at 9kg/tray (fill weights of approx. 50kg/m^2), either as non-supplemented controls or mixed with supplement at the following rates (Table 3):

Table 3: Supplements and their rate of addition used in Trial 2 and Trial 3

Supplement Type	Product Name	Rate of Supplementation (w/w)
Protein	Champfood E *	1.5%
Protein	MCSubstradd Soya	1.5%
Protein	ProMycel Gold *	1.5%
Lipid/protein blend	Natural Gold	1.5%
Carboxylic acid-based	MycroNutrient	0.25%
Mineral-based nutrient	Micromax	0.15%

* At the time of writing the manufacturer's respective web-sites describe ProMycel Gold as *a balanced nutritional content of lipids, carbohydrates, micronutrients, and multiple protein sources* and Champfood to contain vitamins, minerals and trace elements.

The rates chosen for the experiments were based on commercial recommendations and reported rates. In the case of Natural Gold a 1.5% rate was chosen to be consistent (rather than the recommended 1.2%) for comparison with the protein-based supplements. Due to the low inclusion rate of the MycroNutrient and Micromax supplements, these were first suspended in water at 450 and 270 g/L respectively. The water was added to the compost at a rate of 5.6 ml/kg, with the suspended supplement. Water was also added to all the other treatments during mixing at the same inclusion rate.

Trial Three (May/June 2014) was a repeat of Trial Two: Two phase 3 compost types (straw-based and horse manure-based) spawned with A15, supplemented as in Table 3 and with fill weights of approx. 50kg/m^2. In Trial 3 an extra quality parameter was determined, sensory evaluation of the mushrooms, and compost was sampled for gene expression analysis.

Sensory evaluation: From the first flush, 100 or more mushrooms from each supplement treatment (including the non-supplemented control) were harvested and immediately taken to Campden BRI, Chipping Campden for Sensory Evaluation of *fresh* or *raw* mushrooms. From the second flush, similar numbers of mushrooms were taken to Campden BRI for Sensory Evaluation of *cooked* mushrooms.

Gene expression analysis: The straw-based compost treatment was used for compost and mushroom samples for gene expression analysis. A small quantity of compost was sampled at the time of peak mushroom production of flushes 1 and 2 from four replicate trays of the control (non-supplemented treatment), and the three replicate trays from treatments which produced significant increases in mushroom yield i.e. supplementation with protein and with

lipid/protein blend. The compost from one representative protein treatment was chosen for gene expression analysis: Champfood E, and the compost supplemented with Natural Gold, the lipid/protein blend.

The composts were cased with commercial McArdle casing, cacced with phase 3 compost and mushrooms were grown as per commercial conditions.

Data collected and Measurements made

Compost Temperatures

Temperature probes were inserted into the composts to monitor temperature changes after supplementation and during mushroom growth.

Compost Analyses

All samples of composts used in the experiments were analysed for moisture, nitrogen, ammonium-N, and ash contents, pH and electrical conductivity. The moisture content of the composts was also determined at the end of cropping. The supplements were also analysed for moisture and ash contents.

Mushroom Yields

In Trials One, Two and Three the mushrooms were harvested over three flushes and the harvest weights (yields) were measured. The results were analysed by ANOVA.

Mushroom quality (Trials Two and Three) was determined as:

1. Mushroom cap colour using a Minolta meter which measures overall whiteness (parameter 'L') and colour (parameter 'a' for red to green, and parameter 'b' for yellow to blue). Three replicate mushrooms were examined from flushes one and two from each tray. Each mushroom was measured on the top and on two opposite sides.
2. Mushroom cap density (from the non-supplemented compost, and Promycel Gold, Natural Gold and Champfood E treatments) by measuring the weight of a 10mm cube of tissue. Mushroom density is closely correlated with texture or firmness (McGarry and Burton, 1994). Three replicate mushrooms were examined from flushes one and two from each tray.

3. Mushroom Dry Matter content (%): the fresh weight of approx. 150 g of mushrooms was accurately weighed from each tray and for each flush. The mushrooms were then oven dried and the dry weights determined.

4. Mushroom Sensory Evaluation (flavour) conducted at Campden BRI: The mushroom sample preparation prior to was as follows:

   Raw Mushrooms: The mushrooms were gently wiped clean using dry paper towel, stalk ends removed and the mushrooms cut into quarters and placed into two separate coded glass bowls. The samples were then transferred into coded sample containers following the experimental design of the test. Each assessor received two quarter pieces of mushroom per coded container.

   Cooked Mushrooms: The mushrooms were gently wiped clean using dry paper towel and the stalk ends removed. Several baking trays (one tray per mushroom sample variant) were each covered in aluminium foil. Approximately 10ml of vegetable oil was added to each tray and the prepared mushrooms added and mixed until lightly coated in the oil. The trays of mushrooms were placed into a pre-heated fan oven for approximately 10 minutes at 170°C until fully cooked. Once cooked each mushroom was cut into quarters and placed into a pre-heated coded Bain Marie pan to maintain the temperature of the samples throughout the test. To ensure the sensory quality of the mushroom samples was not compromised, a second batch of mushrooms was prepared half way during the panel session. The mushroom samples were served warm (presented as the assessors arrived at the sensory booths) and presented following the experimental design of the test. Each assessor received two quarter pieces of cooked mushroom per coded container.

Sensory Testing

The samples were evaluated using the Triangle Test Procedure (TES-S-001). In the triangle test assessors were presented with a set of three coded samples, two of which were the same and one of which was different. The sets of samples were presented equally often in each of the six possible orders; this experimental design minimised any possible order and carryover effects.

Eighteen trained assessors were used for each test, nine received “test” as the different sample and nine received “control” as the different sample. After tasting the three samples in the designated order, each assessor was asked to select the “different” sample and to describe the difference(s) perceived.

Test Conditions

The test was carried out in a purpose-built testing room. Each assessor was required to undertake the tests in an individual booth. The room was positively pressurised to minimise the entrance of external odours. Coloured lighting (red) was used to mask any colour difference between the samples. The panel used water crackers and filtered water as palate cleansers between the samples to minimise sample carry-over.

Gene Expression analysis of composts:

A total of 24 compost samples were collected (three treatments (control, Champfood E and Natural Gold) × two flushes × four replicates).

After harvest, the composts were frozen in liquid nitrogen and transported frozen to East Malling Research where samples were stored at -80^0 C until use. RNA was extracted from the compost using a method developed in the MushTV project; RNA extraction from mushroom compost involved milling the frozen compost using metal ball bearings, extraction of RNA with Trizol buffer followed by a clean-up step using silica. Each purified RNA sample was converted by random priming into cRNA via cDNA, fluorescently-labeled with cyanine-3, and hybridised to a different microarray. To determine the amount of hybridiation to each probe, arrays were laser scanned and the resulting images analysed using commercially available image analysis software. The microarrays were manufactured by Agilent Technologies (Santa Clara, California, USA) incorporating 10468 *in situ* synthesized 60-mer probes. Each probe was replicated five times on each microarray.

Data handling

The data were imported into 'R' where normalisation, filtering and statistical analysis were undertaken using the Bioconductor Limma package (Ritchie *et al*., 2015).

Normalisation

The data were background corrected using normal exponential convolution with an offset of 50. Between array normalisation was performed using the quantile method.

Data filtering

To reduce false discovery rate of differentially expressed genes, the data were filtered to remove features that showed no expression (using the Agilent Well Above Background Boolean flag).

Each biological replicate set was filtered using an aggressive filter (≥ 50% present) due to the small number of biological replicates (<=four) for each set (McClintick and Edenberg, 2006). Features failing the filter across all sets were removed before calculating significance. Control probes were removed prior to normalisation.

Outliers within a technical replicate set were replaced with the median value of that set. Outliers were defined by two criteria: (1) log expression value greater/less than 1.0 from set median value and (2) replicate set range greater than 2.0. Technical replicates were then combined and log expression value replaced with the set median value.

Statistical analysis

Initial analysis identified two arrays as “outliers” with very different expression patterns compared with comparable arrays, these were then excluded from further analyses.

For statistical analysis and normalisation the data from the remaining 22 microarrays were combined. Comparisons were made between treated and control samples, with each time-point taken separately. Differences in transcript levels between the comparisons were calculated using Empirical Bayes Statistics for Differential Expression (Smyth, 2004) with Benjamini and Hochberg False Discovery Rate correction for multiple testing (Benjamini and Hochberg, 1995). Statistical significance was taken as $p <= 0.05$.

GO functional analysis

GO (Gene Ontology) functional analysis was performed using the BiNGO plugin (2.44) for Cytoscape 2.8.3 (Maere *et al*., 2005). Gene Ontology was downloaded from the Gene Ontology Consortium (geneontology.org/page/downloads) on 07/07/2015. An *A. bisporus* gene to GO annotation file was created manually from publically available *A. bisporus* functional annotation.

Statistical Analyses of Yield and Quality Data

For trials 2 and 3 there were three replicate trays per treatment/compost type combination and six replicate trays for the non-supplemented controls. To account for any within house variation, the replicate trays were distributed in the mushroom house in replicate blocks. For Trial One, which examined the effects of supplements on Heirloom growing on straw-based compost, three replicate trays were used per supplement treatment. However comparisons cannot be made with A15 performance as only two trays of A15 growing on a horse manure-based phase 3 compost were grown

Yield data and quality data were analysed by Analysis of Variance (ANOVA) using Genstat unless stated otherwise.

Results

The review of nutritional supplement usage

Numerous conversations have been held with mushroom growers, compost manufacturers and supplement suppliers concerning usage of nutritional supplements. The situation is dynamic with growers and composters prepared to change or look at different supplements, the partial replacement of soy with keratin-based products and the recent interest in liquid casing supplements.

The Key findings of these conversations are:

- >90% of phase 3 compost in Europe is supplemented because it is believed that supplement use has a beneficial effect on quality and production of mushrooms.
    - Less supplementation during summer
    - Some Heirloom growers have reduced supplement usage particularly in the summer. A large Pennsylvania grower has informed the authors that in his experience with trialling supplements with Heirloom, only a small and variable increase in yields was detected.
    - Protein supplement most commonly used
    - Rate: 1.4 – 1.5% for protein supplements, 1.2% for Natural Gold

- Protein from soy and other protein products:
    - soy
    - soy + various formulations (alternative protein sources and minerals)
- NutriGain products (lipid/protein blend and carboxylic acids) in commercial use
    - Cracked maize (Poland)
- There is considerable interest in the potential of liquid supplement for compost and/or casing

A list of available supplements is given in the table (Table 4) below:

Table 4: List of available supplements. The ingredients and the proportion of protein in the supplements can vary with time. The products and their compositions are accurate at the time of writing **(**the rate of application is given for phase 2 (Ph 2) and phase 3 (Ph 3) composts)

Company	Product	Ingredient	Protein %	Moisture %	Rate % w/w
Lambert	T6	feathermeal+ polysaccharide	44	Unknown	0.8 (Ph 2)
Lambert	T7	feathermeal+ polysaccharide	66	Unknown	0.8 (Ph 2) <0.8 (Ph 3)
Lambert	S44	soya	44	Unknown	?
Amycel	ProMycel Gold	soya + feathermeal	54	7	1.5 (Ph 3)
Amycel	PROCO 50 (a new product)	rape meal + soya + trace elements	50	11	1.5 (Ph 3)
MCSubstradd (Havens)	"Protein-cocktail"	feathermeal, soya, veg. proteins, rape meal	44	12	≤1.5 (Ph 3)
MCSubstradd (Havens)	Soybean based	soya	?	Unknown	≤1.5 (Ph 3)
Champfood	Champfood C	soya, animal protein, rape	48	Unknown	≤1.5 (Ph 3)
Champfood	Champfood E and EXC	soya, animal protein, rape meal, minerals	48	12	≤1.5 (Ph 3)

Company	Product	Ingredient	Protein %	Moisture %	Rate % w/w
NutriGain	MycroNutrient	citric acid, propionic acid	0	24	casing application
NutriGain	Natural Gold	Lipid/Protein blend	0	11	0.8 – 1.4 (Ph3)
Everris	Micromax	Ca (12%), Mg (3%), S (12%), B (0.1%), Cu (1%), Fe (17%), Mn (2.5%), Mo (0.05%), Zn (1%)	0	2	
Aril (Poland) http://www.aril.pl/	Podloza Faze III Standard	starch	?	?	0.7 – 2.0 (Ph3)

Effect of Compost Supplements on Heirloom mushrooms (Trial One)

Compost temperatures

Compost temperatures of the different treatments were measured and shown in Figure 1. Supplementation with Micromax or MycroNutrient did not change the compost temperatures compared with the non-supplemented control. However, supplementation with the protein-based supplements (ProMycel Gold, Champfood E, MCSubstradd and Natural Gold) did cause a 'spike' or rise in compost temperature compared with the non-supplemented control, after about 2 days.

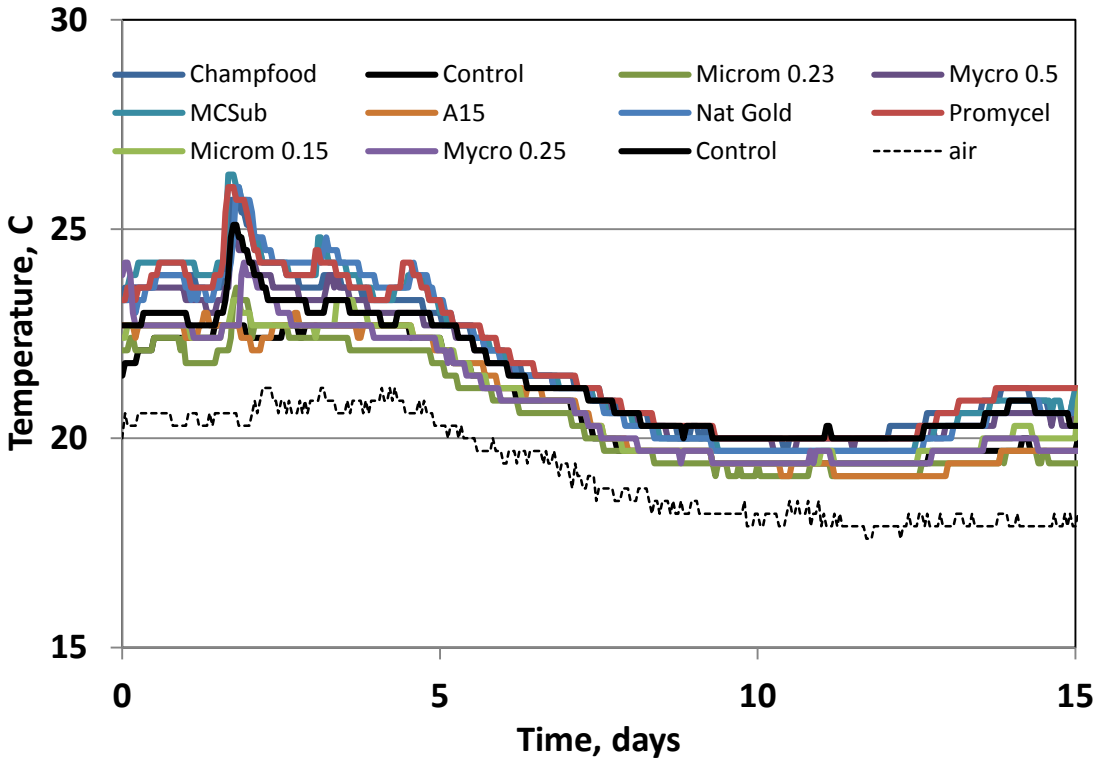

Figure 1: The effects of supplementation on Phase 3 compost temperatures (*A. bisporus* strain: Heirloom) after supplementation (at time 0)

Effects of supplements on mushroom yield

The effects of supplements on mushroom yield over three flushes for Heirloom strain is shown below in Figure 2.

Non-supplemented phase 3 compost produced a yield equivalent of Heirloom mushrooms of 271 kg/tonne. The supplementation with MCSubstradd soya increased the yield significantly to 368 kg/tonne. The increase in yield can be seen to come from all three flushes (Fig. 2). Supplementation with ProMycel Gold, Champfood E, Natural Gold, MycroNutrient and Micromax had no significant effect on yield.

By way of comparison, strain A15 (non-supplemented) produced a yield of 321 kg/tonne.

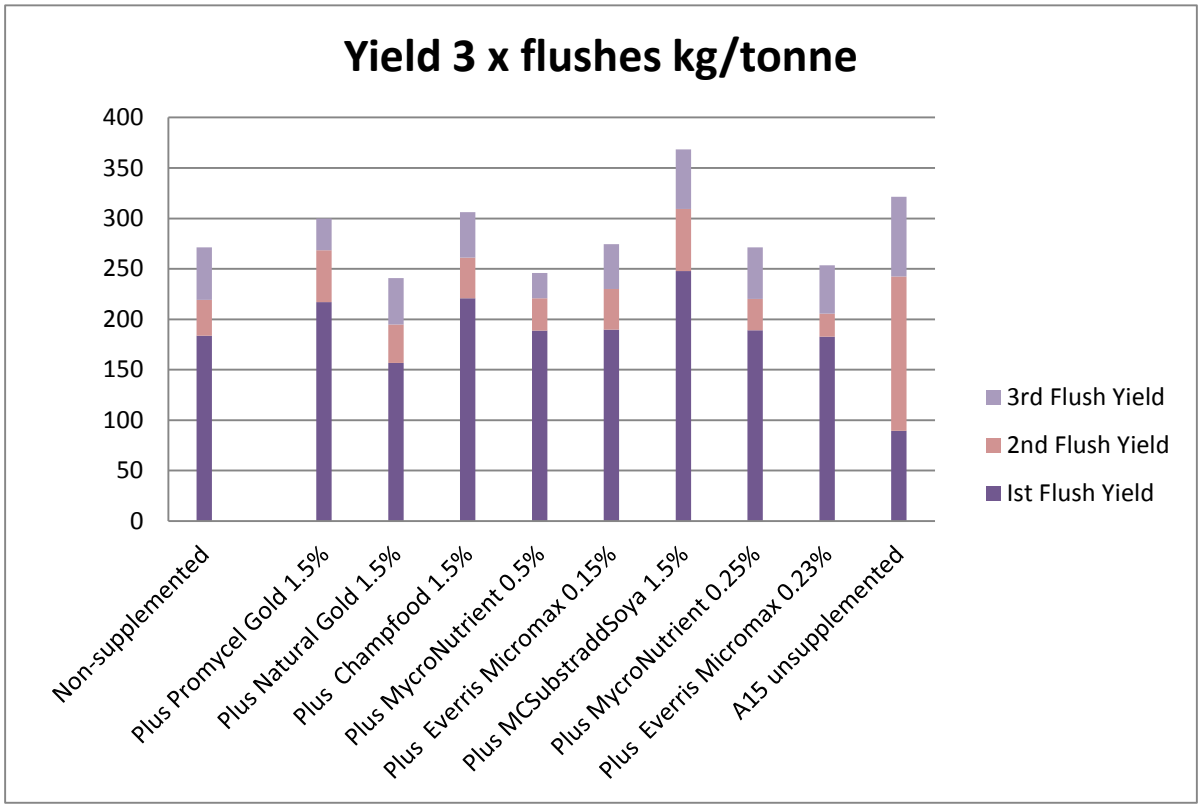

Figure 2: The effects of supplements on three flushes of mushroom yield of Heirloom strain from Trial One

The main purpose of this proving trial was to test out experimental procedures and a new test cropping facility. However, the work does suggest that one of the protein-based supplements (MCSubstradd) can significantly benefit yield of a brown strain, Heirloom. This will be of interest to growers of brown strains and deserves more detailed attention in the future. The lack of yield increase by the other protein-based supplements is surprising.

Percentage Dry Matter

The dry matter contents (%) of the mushrooms were determined and are shown in Figure 3. No significant difference between the treatments was found.

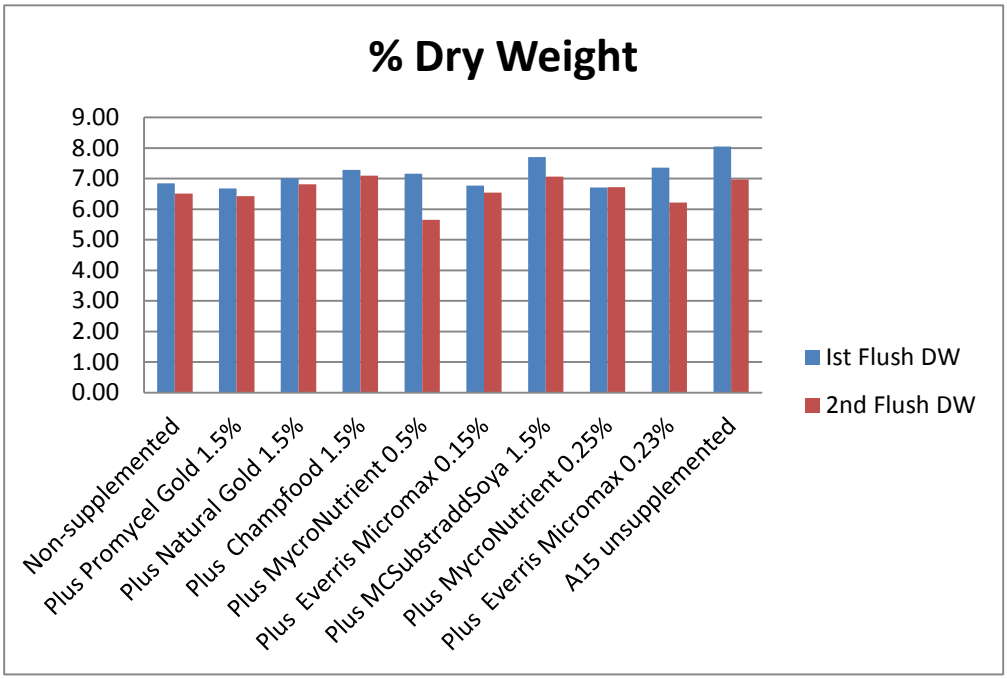

Figure 3: The dry matter content (%) of Heirloom mushrooms from Trial 1

Effect of Compost Supplements on A15 mushrooms (Trials 2 and 3)

Unless stated otherwise, the results for Trials 2 and 3 were combined before being statistically analysed together

Compost Temperatures

Temperature probe read-outs from Trial 2 are shown in Figures 4 and 5. None of the supplement treatments resulted in very high temperature spikes. Champfood and Natural Gold treatments resulted in the highest compost temperatures, up to 3C higher than the unsupplemented control treatment. The MCSubstadd supplement increased compost temperature by 1-2C above the control treatment.

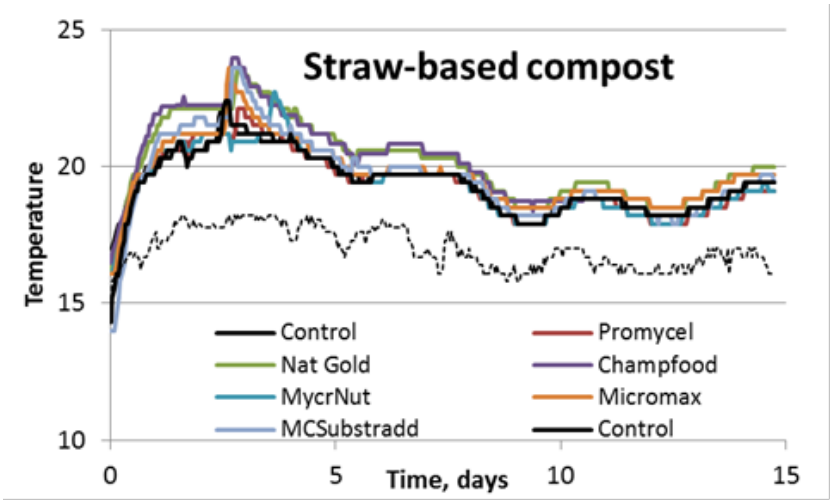

Figure 4: The effects of supplementation on the temperatures of phase 3 straw-based compost (*A. bisporus* strain: A15) after supplementation (at time 0)

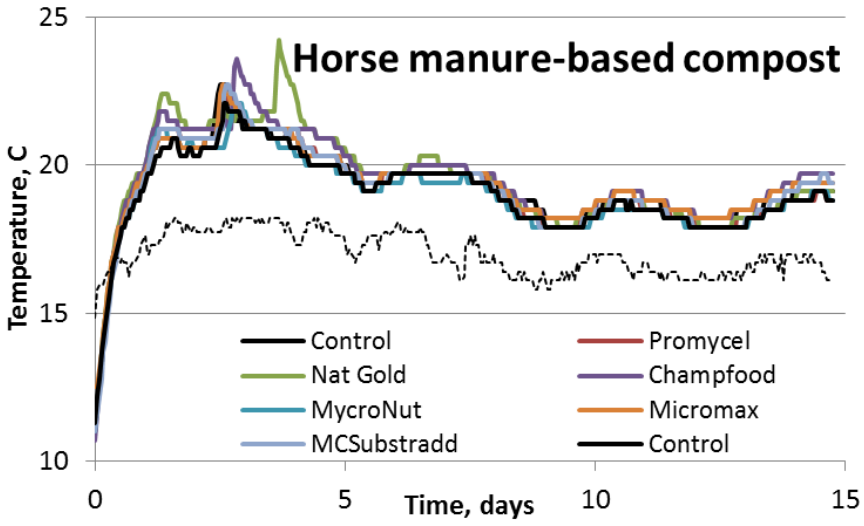

Figure 5: The effects of supplementation on the temperatures of phase 3 horse-manure based compost (*A. bisporus* strain: A15) after supplementation (at time 0).

Table 5: Analysis of composts used in the trials

Trial	Compost type	mg/kg compost dry mat.			moisture	pH	EC
		Total N	NH^4-N	Ash	% w/w		mS/cm
1	Horse manure	2.35	0.13	-	64.3	6.16	6.55
1	Straw-based	2.35	0.14	25.0	68.1	6.00	5.48
2	Horse manure	2.15	0.12	32.3	64.4	6.11	6.72
2	Straw-based	2.64	0.14	23.6	66.0	6.22	4.97
3	Horse manure	2.12	0.10	31.8	59.9	6.11	7.98
3	Straw-based	2.14	0.10	20.0	62.7	6.36	7.96

The horse manure composts had higher ash, dry matter and electrical conductivities (EC) than the straw-based composts (Table 5). The ammonium nitrogen (NH_4^+) content and pH values of the two composts were similar. The straw-based compost in Trial 2 had a higher nitrogen content than the other batches of composts used in the trials. The horse manure composts still had higher ash and dry matter contents than the straw-based composts at the end of the third flush of mushrooms (Table 6).

Table 6: Analysis of composts after the third flush in the trials

Trial	Compost	Ash	moisture	pH	EC
		mg/kg d.m.	% w/w		mS/cm
1	Horse manure	-	-	-	-
1	Straw-based	28.4	64.5	7.11	6.44
2	Horse manure	40.8	61.9	6.25	7.22
2	Straw-based	27.7	66.1	6.12	5.48
3	Horse manure	39.4	63.6	6.15	5.55
3	Straw-based	31.8	66.1	6.31	4.82

Table 7: Analysis of supplements

Supplement	Moisture, %	Ash, % of dry matter
Promycel Gold	5.7	8.9
Champfood	11.5	8.4
MCSubstradd	12.3	11.3
Natural Gold	10.6	8.0
MycroNutrient	24.4	31.7
Micromax	1.6	73.7

Micromax had a higher ash content and lower moisture content than the other supplements used in the trials (Table 7). Of the remaining supplements, MycroNutrient had the highest moisture and ash contents (before addition of water) and Promycel Gold had the lowest moisture content.

Mushroom Yield

The effects of supplementation on mushroom yields are shown in Figure 6 and Table 8 (same data). These means are from the combined yield data from Trials 2 and 3 and combined from both compost types; straw-based and horse-manure based.

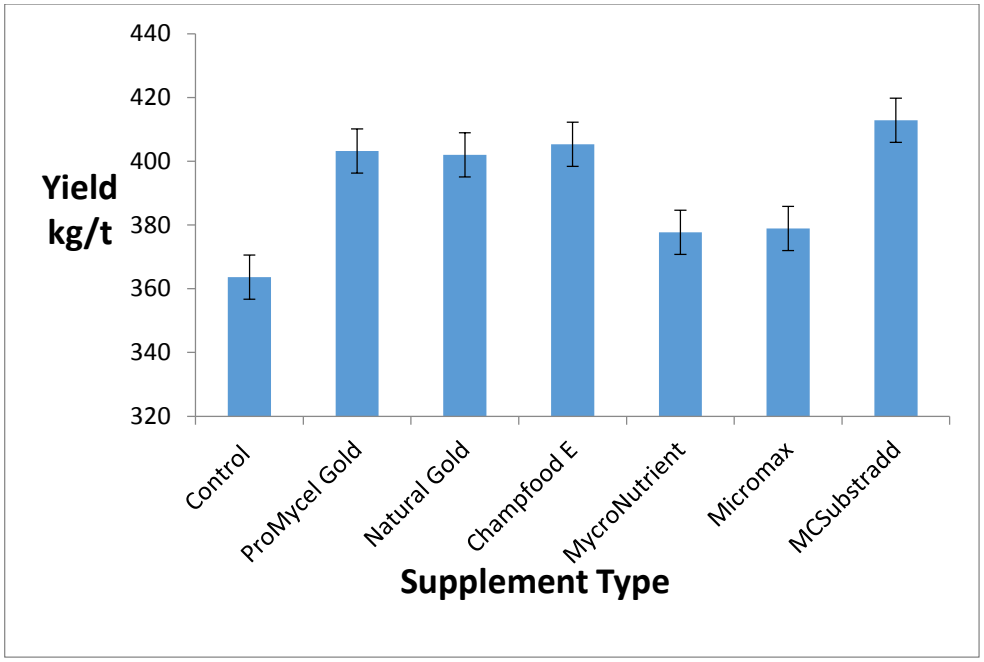

Figure 6: The effects of supplementation on mushroom yield of A15 mushrooms from Trials 2 and 3. Error bars show the Standard Error

Table 8: The effects of supplementation on mushroom yield (kg/t) of A15 mushrooms from Trials 2 and 3 (same data as Figure 6)

Control	ProMycel Gold	Natural Gold	Champfood E	Mycro Nutrient	Micromax	MC Substradd
363.6	403.2	402	405.3	377.7	378.9	412.8

The control non-supplemented composts produced an average yield of 363.6 kg/t. Supplementation with the protein and lipid-protein products significantly increased yields ($p < 0.05$) to 402-412 kg/t. The average increase in yield by the four supplements containing protein was 11.5%. There were no significant differences in the yields between protein-based supplements. There was no significant yield effect of supplementation with MycroNutrient or Micromax. It should be emphasized that MycroNutrient is marketed as a casing supplement, it was included here to make the comparisons of compost supplements complete.

Effect of compost-type on mushroom yields

Trials 2 and 3 both used two different compost types; straw-based and horse manure based. No significant differences were found between the compost types on mushroom yield. However, batch to batch variability was detected as Trial 2 showed a greater yield for horse manure based compost and Trial 3 showed a greater yield for straw based compost (Figure 7). This was possibly due to the higher moisture content of the straw-based composts. In Trial 3, this was beneficial (62.7% for straw-based compost and 59.9% for horse manure compost). However, in Trial 2, this may have been detrimental (66% for straw-based compost and 64.7% for horse manure compost).

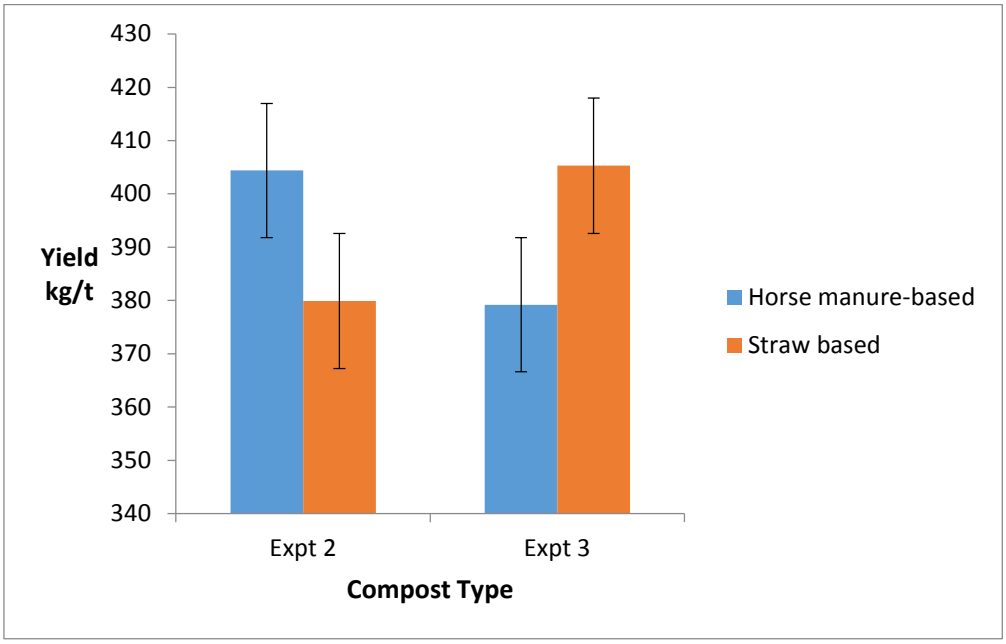

Figure 7: The effects of compost-type on mushroom yield of strain A15 from Trials 2 and 3. Error bars show the Standard Error.

Mushroom Quality

Mushroom quality was measured as: mushroom cap colour, mushroom density, dry matter content (%) (all combined for Trials 2 and 3) and mushroom sensory analysis (Trial 3 only).

Mushroom cap colour:

Mushroom cap colour was measured using a Minolta meter which produces three parameters to describe colour: L, a measure of the lightness to darkness, pure white being a figure of 100; 'a' a measure of redness; and 'b' a measure of yellowness. Cap colour was

measured on the tops and the sides of the mushroom as these are known to have different colour characteristics (Burton, Frost and Atkey, 1987).

L, a measure of the lightness to darkness: No clear effect of supplement on the L value of mushroom colour was detected. However, mushrooms grown on straw-based compost were found to be significantly whiter than horse-manure based compost in both tops and sides of flush one and the tops of flush two mushrooms (Figure 8).

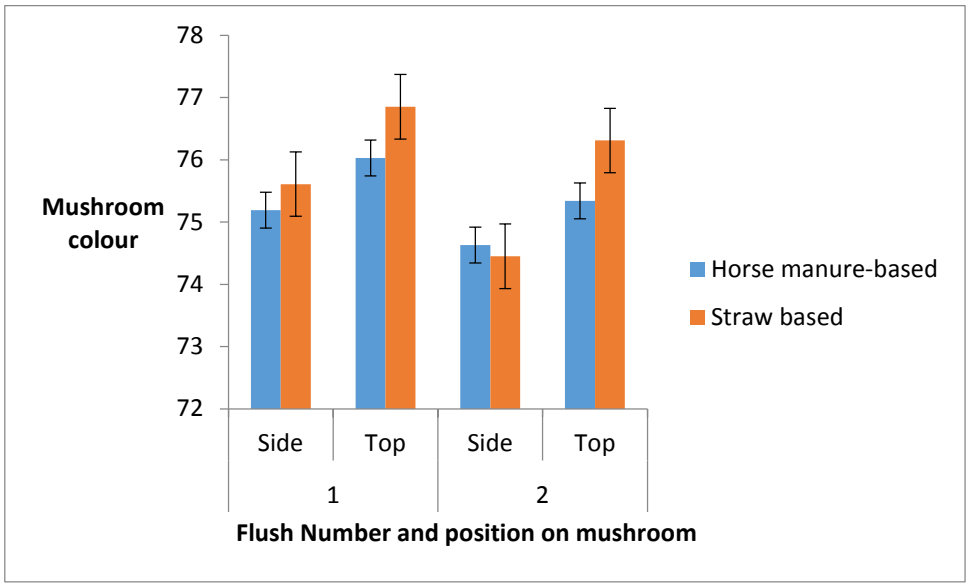

Figure 8: The effects of compost-type and flush number on the 'L' value on the colour of mushroom caps, tops and sides. Error bars show the Standard Error

The 'a' measure of colour – redness

The 'a' measure of colour – redness of the mushrooms was found to be very close to zero and so not useful for analysis or interpretation.

The 'b' measure of colour – yellowness

Evidence was found that two of the supplements (ProMycel Gold and McSubstradd) led to a small but significant ($p < 0.05$) increase in yellowness when data are averaged across all treatments (Figure 9). However, the small effects of supplements on mushroom yellowness appear to be affected by compost-type and flush number (figures 11 and 12). Therefore, it is difficult to be sure of an overall trend.

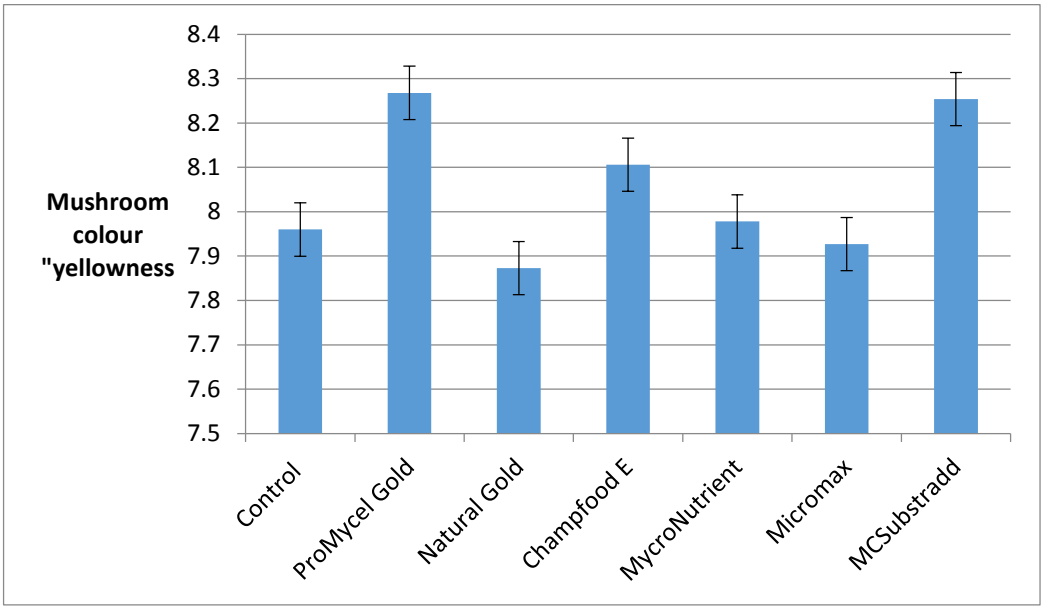

Figure 9: The effects of supplements on the yellowness ('b' value on the colour) on mushrooms (averaged across Trials 2 & 3, flushes and compost-type). Error bars show the Standard Error.

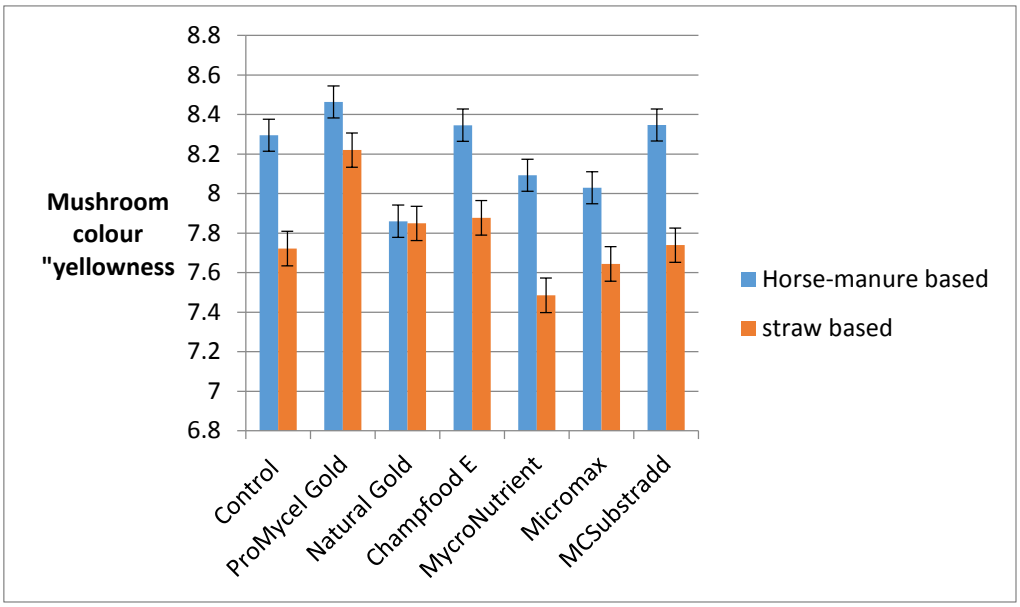

Figure 10: The effects of supplements on the yellowness ('b' value on the colour) on first flush mushrooms. Error bars show the Standard Error.

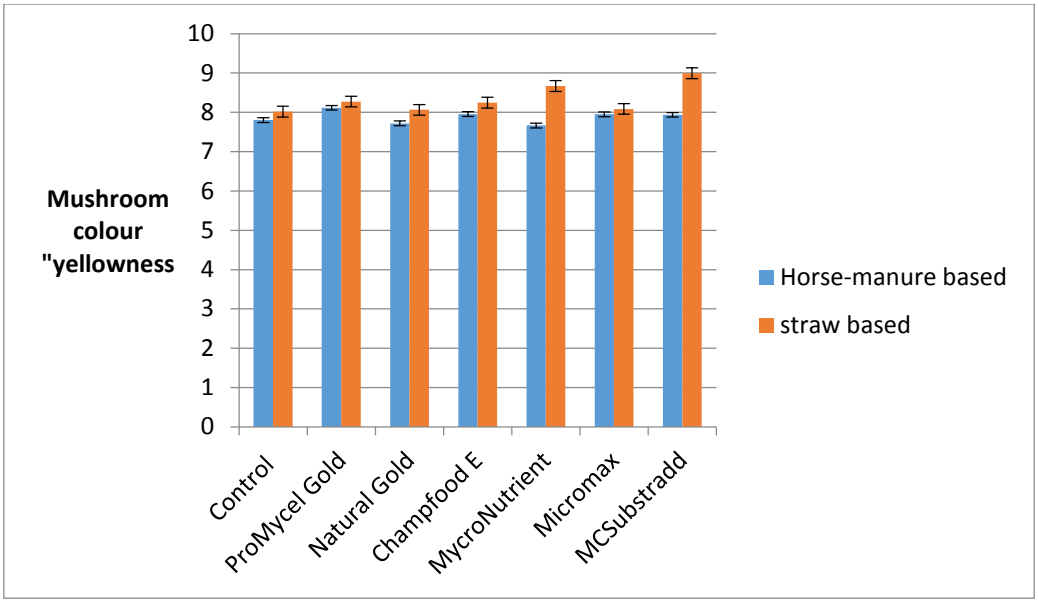

Figure 11: The effects of supplements on the yellowness ('b' value on the colour) on second flush mushrooms. Error bars show the Standard Error

Mushroom density

The effects of four supplement treatments (control (non-supplemented), Promycel Gold, Natural Gold and Champfood E) and the two compost types on mushroom cap density were measured.

Promycel Gold and Champfood E supplementation significantly increased mushroom density by an average of 5.5% ($p < 0.002$), Figure 12.

Mushrooms grown on horse manure-based compost were consistently and significantly ($p < 0.05$) more dense than those grown on straw-based compost (Figure 12).

Cap density has been shown to directly relate to cap texture (McGarry and Burton, 1994) so this increase in density equates to an improvement in texture. Also increased density means a higher picking rate i.e. more weight for a mushroom of the same size.

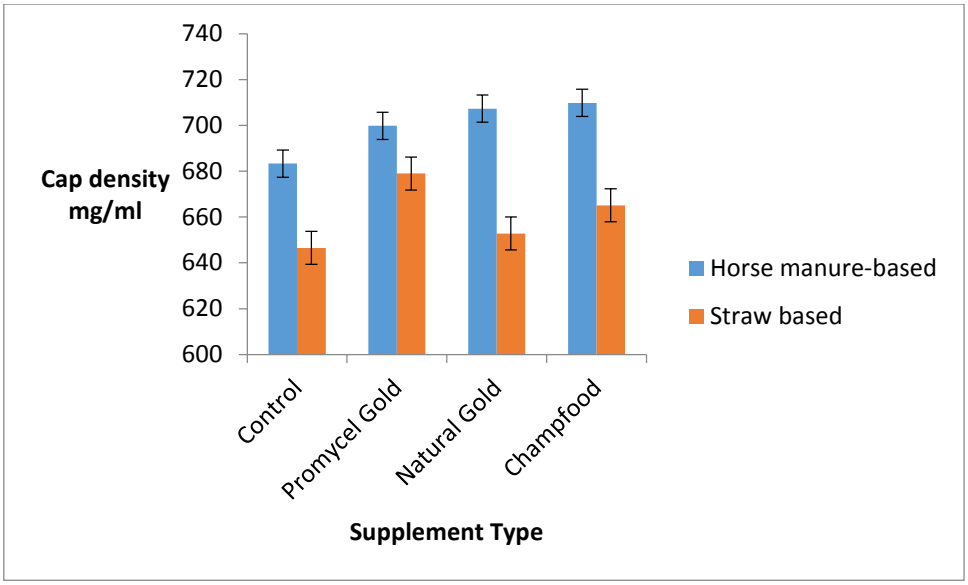

Figure 12: The effects of supplements and compost-type on mushroom density. Error bars show the Standard Error.

Dry matter Content (%)

There were no significant differences of supplement (p = 0.44) use or compost type (p = 0.066) in the dry matter content (%). First flush mushrooms had a dry matter content of 7.834% while second flush mushrooms it was 7.094%.

Mushroom Sensory Analysis

Mushroom samples were sent to Campdens BRI for sensory evaluation to determine whether nutritional supplementation could affect flavour and any other sensory aspects of the mushrooms. Mushrooms provided to Campdens BRI were (1) control non-supplemented; and mushrooms supplemented with (2) Mycronutrient; (3) Champfood (as an example of the three protein supplements); (4) Micromax and (5) Natural Gold. Two sets of evaluations were performed on fresh or raw mushrooms and on cooked mushrooms.

The test performed was the Triangle Test e.g. whereby assessors were given (blind) two control mushrooms and one supplemented mushroom, or vice versa, and try to identify the 'odd one out'. Using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance.

Results

No evidence was found that any of the supplement treatments affected the sensory properties of mushrooms (Table 9). The full report from Campden BRI is in Appendix One of this report.

Table 9: The effects of supplementation on mushroom sensory qualities. Mushrooms, grown on four supplements, were compared to control (non-supplemented mushrooms) as raw/fresh mushrooms and as cooked mushrooms

Supplement Type	Mushrooms Test Raw/Fresh Or Cooked	Number of Assessors	No. Correctly Identifying the Different Sample	Significance
Mycronutrient	Raw/fresh	18	5	NSD*
	Cooked	18	9	NSD*
Champfood	Raw/fresh	18	6	NSD*
	Cooked	18	7	NSD*
Micromax	Raw/fresh	18	7	NSD*
	Cooked	18	7	NSD*
Natural Gold	Raw/fresh	18	6	NSD*
	Cooked	18	5	NSD*

* NSD: Not Significantly Different at the 5% alpha (α) level of significance

An example to understand the Campden BRI document:

For the mushrooms grown on Mycronutrient supplemented compost and assessed as cooked, 9 assessors identified a mushroom type as ‘different’. This could be considered to be an interesting result as the number is just below the threshold number of 10 assessors required to establish a significant difference the sensory quality of the mushrooms. However, if one then examines the details of the differences described (Table 10) one can then discover that these differences are not all consistent and so do NOT point to a ‘near-miss’ in significance. For example, one assessor considered the control mushrooms to be “more bitter” and another assessor considered the Mycronutrient-supplemented mushroom to be “more bitter”.

Table 10: Assessor comments on the effects of Mycronutrient supplementation on mushroom sensory qualities. six assessors identified the control mushrooms to be different and three assessors found the Mycronutrient mushrooms to be different. However, some of these comments are contradictory and so the overall conclusion is that there is no effect of this supplement on flavour

Mushroom type identified as 'different'	Individual comments (and numbers giving those comments)
Control or non-supplemented mushrooms	More bitter (1) Slight metallic taste (1) Fresher taste (1) Much softer texture and stronger perfumed note (1) Very little difference between the samples, this sample was juicier and stronger in flavour (1) No difference (1)
Mushrooms grown on Mycronutrient supplemented compost	Bitter and metallic taste (1) More bland, less sharp aftertaste (1) Slightly sour aftertaste, less sweet (1)

Overall conclusion of the sensory assessments is that there is no evidence that the use supplements can change mushroom flavour or other sensory properties.

A. bisporus Mycelial Gene Expression

To gain insights into the mechanism of how supplements can increase mushroom yields, gene expression analysis was conducted on compost samples using microarrays. Four replicate samples of compost were collected at two time points (mid first flush and mid second flush), from the control (non-supplemented) trays and from trays supplemented with Champfood E (representative of the protein treatment) and from Natural Gold (the lipid/protein blend treatment).

The summary of this work is as follows and a fuller scientific examination of the results is provided below that

Summary:

- No differences were found in gene expression between the supplement treatments for second flush compost samples
- Only one gene was found to be different in the Champfood-supplemented compost, first flush (compared with non-supplemented control compost). This gene was down-

regulated (less being made); however the function of the protein encoded by this gene has not yet been elucidated. This is a surprising result as the protein supplements caused large and significant increases in yield. A possible explanation is that when *A. bisporus* grows in non-supplemented compost it produces sufficient enzymes (to release the nutrition from the compost) which have capacity to take on and absorb the extra protein available in the Champfood-supplemented compost

- Supplementing with Natural Gold (the lipid/protein blend) caused six genes to be up-regulated and 21 genes to be down-regulated compared with non-supplemented control compost (first flush). This suggests that the Natural Gold elicits a different response from non-supplemented compost and the protein-only supplemented compost. The up-regulated genes were involved in nitrogen and carbon metabolism. Six of the down-regulated genes are involved in lipid metabolism. The conclusion here is that the Natural Gold is having an additional response on the *A. bisporus* metabolism via enzymes produced compared with non-supplemented compost and supplemented with protein alone.

More Detailed analysis of Gene Expression experiments

Initial analysis identified two microarrays as “outliers” with very different expression patterns compared with comparable microarrays (Figure 13), these were then excluded from further analyses.

The statistical analysis indicates that for Flush 2 there were no significant differences in the gene expression between the supplement treatments. Also large differences were found in gene expression between first and second flush composts (2,500 genes differentially regulated in non-supplemented samples between the first and second flushes, ($p < 0.01$)). This suggests that the profile of genes (and therefore enzymes) being synthesised in the compost mycelia are very different between the two flushes and also probably within flushes.

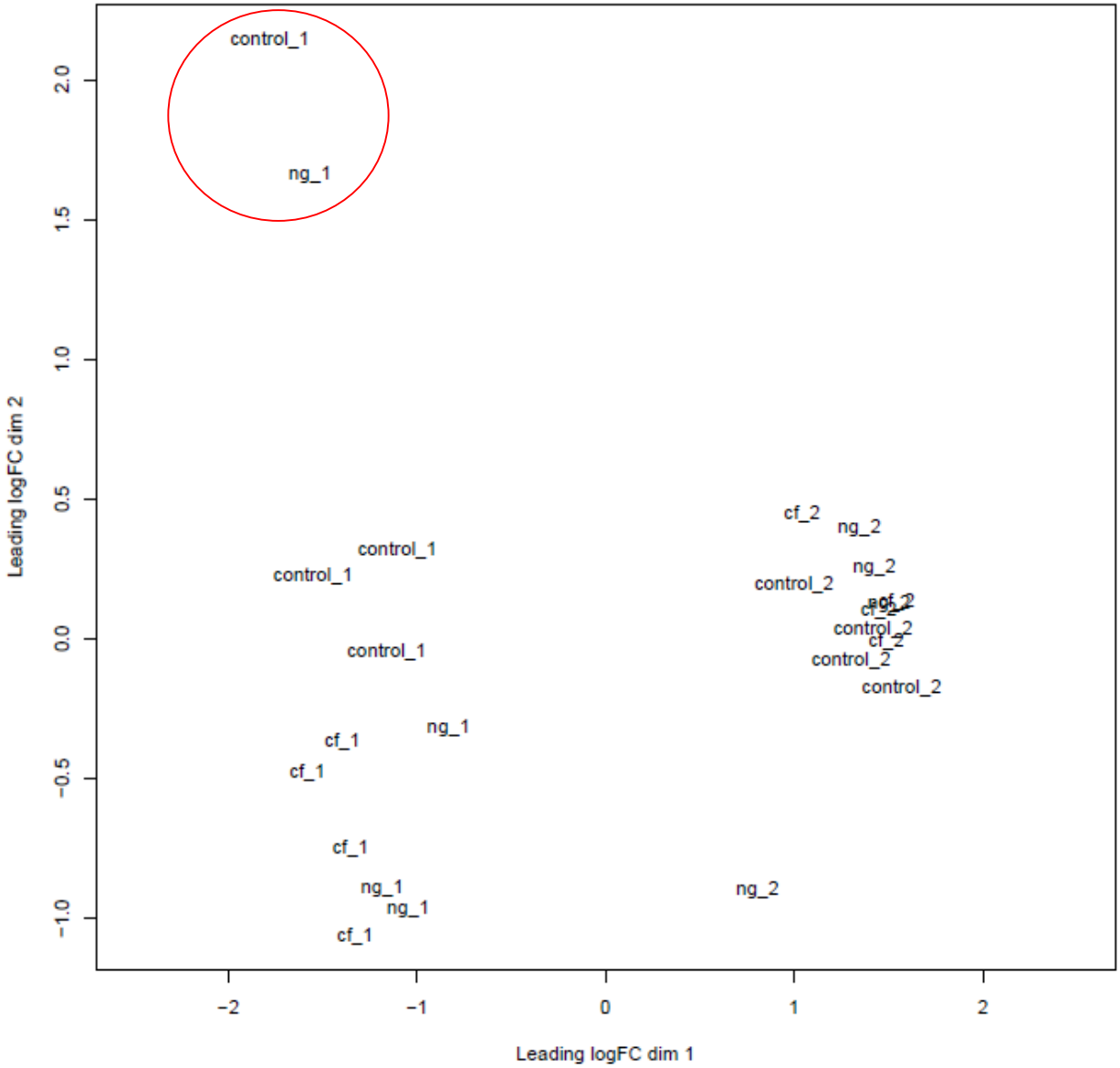

Figure 13: Multidimensional scaling plot (Principal Component Analysis) of 1st and 2nd flush microarray data. Circled samples were excluded from analysis. Individual plots are labelled as supplement type (control; cf = Champfood; ng = Natural Gold) and flush numbers (flush 1 and flush 2)

However, for the first flush statistically significant differences in gene expression were found between treatments.

First Flush: Champfood vs. Control (non-supplemented)

First flush compost containing Champfood had one gene down-regulated to 29.1% (significance: $p < 0.03$) compared to the control. This gene is described by the identifier

'KV42663' but there's no functional annotation for this gene i.e. we don't know what it codes for.

First Flush: Natural Gold vs. Control (non-supplemented)

First flush compost supplemented with Natural Gold had 6 genes up-regulated compared with the control (Table 11) and 21 genes down-regulated compared with the control (Table 12) (statistical significance: $p <= 0.05$).

The six up-regulated genes in Natural Gold compost indicate an increased metabolism particularly nitrogen and sugar utilisation (Table 11).

Glutamate dehydrogenase and glutamine synthetase (Table 11) are enzymes involved in nitrogen metabolism, possibly ammonia accumulation. The enzyme, hexokinase, functions in sugar utilisation and carbon source sensing. Coproporphyrinogen oxidase is an enzyme on the haem synthesis pathway; haem-proteins have a wide range of functions. Argininosuccinate lyase is a key enzyme on the energy releasing pathway (Citric acid cycle) at a branch point with the urea cycle (a nitrogen metabolism cycle).

The 21 down-regulated genes in Natural Gold compost are shown in Table 12. Genomic or transcriptomic analyses such as these are usually examined bioinformatically to draw out trends or patterns to comprehend the change in biology.

Bioinformatic analysis (Table 13) revealed that 100% of the down-regulated genes code for proteins with catalytic function involved in metabolic processes which is significantly different proportion from all genes of the *Agaricus bisporus* genome ($p < 0.01$ and $p < 0.04$ respectively). There is a down-regulation of three genes involved in lipid synthesis (Table 13: genes in rows 7, 8, and 9) ($p < 0.04$); and 3 genes involved in oxidation pathways of lipids (Table 13: genes in rows 13, 14, and 17) ($p < 0.04$).

Clearly, the Natural Gold supplement has stimulated a different response in gene expression. Analysis of the individual genes reveals that there is increased activity (as in gene expression) towards nitrogen activity and decreased activity towards lipid metabolism. This is interesting as Natural Gold is a lipid/protein blend. The supplement appears to increase capacity of *A. bisporus* to metabolise the nitrogen (presumably from the added protein) but changed the profile of lipid metabolism genes which suggests that the *A. bisporus* mycelium is responding and utilising the additional lipids from the supplement.

Table 11: Up-Regulated genes showing significant (p<=0.05) differential expression in Natural Gold treated 1st Flush mushroom samples compared to control samples

Gene ID (EBI)	% of control value	*p*	Description
EKV45647	361	0.05	Glutamate dehydrogenase
EKV43005	228	0.04	Predicted glutamine synthetase
EKV43879	211	0.04	Hexokinase
[1]EKV46555	191	0.03	Coproporphyrinogen oxidase
[1]AW444402/EKV46555	191	0.04	Coproporphyrinogen oxidase
EKV50172	189	0.04	Argininosuccinate lyase
EKV50704	164	0.04	Unknown

[1] Same gene (EKV IDs are from genome project other IDs from other projects)

Table 12: Down-Regulated genes showing significant (p<=0.05) differential expression in Natural Gold treated 1st Flush mushroom samples compared to control samples

Gene ID (EBI)	% of control value	*p*	Description
EKV41797	59	0.05	Unspecific monooxygenase
EKV42890	58	0.05	Exoribonucleases producing 5'-phosphomonoesters
EKV49956	54	0.05	Enoyl-CoA hydratase
EKV47943	50	0.05	Short-chain acyl-CoA dehydrogenase
AW444420/EKV43696	48	0.04	Arylacetamide deacetylase
EKV47171	47	0.02	2-methylcitrate dehydratase
EKV45111	47	0.05	DNA-directed RNA polymerase
EKV50672	45	0.04	3-oxoacyl-[acyl-carrier protein] reductase.
AW324531/EKV44017	43	0.05	isocitrate dehydrogenase (NADP+)
EKV51564	42	0.02	FAD dependent oxidoreductase/ Microfibrillar-associated protein MFAP1
EKV50887	39	0.05	Enoyl-CoA hydratase
EKV49172	36	0.02	Glutathione peroxidase
EKV47722	35	0.03	Unknown (DUF1479)
EKV51314	32	0.04	Acetyl-CoA C-acyltransferase
EKV49647	32	0.04	3-oxoacyl CoA thiolase
EKV44080	32	0.05	AAA ATPase containing von Willebrand factor type A
GH158931/EKV48765	32	0.02	4-coumarate CoA ligase
EKV47243	25	0.05	Succinate dehydrogenase
EKV47643	25	0.03	Protein kinase-like
EKV42663	23	0.01	Unknown
[2]EKV49318	20	0.02	Predicted NAD-dependent oxidoreductase
EKV44345	18	0.05	3-oxoacyl-[acyl-carrier protein] reductase
EKV45917	17	0.02	IMP dehydrogenase
[2]AW444428/EKV49318	16	0.04	Predicted NAD-dependent oxidoreductase
[2]AW444427/EKV49318	16	0.04	Predicted NAD-dependent oxidoreductase
EKV50651	10.4	0.05	L-arabinose isomerase

[1] Same gene (EKV IDs are from genome project other IDs from other projects)
[2] Same gene

Table 13: GO (Gene Ontology) functional analysis of GO annotated genes with significant down regulated expression in Natural Gold treated 1st Flush samples compared to control samples, *i.e.* down regulated genes in table 12. P is Benjamini-Hochburg corrected for false discovery rate. Cluster freq. is the number of genes containing the GO-ID for down regulated genes in table 12. Total freq. is the number of *Agaricus bisporus* genes containing the GO-ID in the total of GO annotated *A bisporus* genes

	GO Description	GO-ID	*p*	cluster freq.	total freq. in *A. bisporus* genome
1	catalytic activity	3824	0.01	21/21 100.0%	3152/5039 62.5%
2	oxidoreductase activity	16491	0.03	10/21 47.6%	757/5039 15.0%
3	oxidation-reduction process	55114	0.03	10/21 47.6%	766/5039 15.2%
4	metabolic process	8152	0.04	21/21 100.0%	3572/5039 70.8%
5	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	16616	0.04	4/21 19.0%	118/5039 2.3%
6	oxidoreductase activity, acting on the CH-CH group of donors	16627	0.04	3/21 14.2%	61/5039 1.2%
7	3-oxoacyl-[acyl-carrier-protein] reductase (NADPH) activity	4316	0.04	2/21 9.5%	18/5039 0.3%
8	fatty acid synthase activity	4312	0.04	2/21 9.5%	19/5039 0.3%
9	short-branched-chain-acyl-CoA dehydrogenase activity	16937	0.04	1/21 4.7%	1/5039 0.0%
10	propionate metabolic process	19541	0.04	1/21 4.7%	1/5039 0.0%
11	propionate catabolic process	19543	0.04	1/21 4.7%	1/5039 0.0%
12	propionate catabolic process, 2-methylcitrate cycle	19629	0.04	1/21 4.7%	1/5039 0.0%
13	very-long-chain-acyl-CoA dehydrogenase activity	17099	0.04	1/21 4.7%	1/5039 0.0%
14	propionyl-CoA dehydrogenase activity	43820	0.04	1/21 4.7%	1/5039 0.0%
15	thiol-driven fumarate reductase activity	43830	0.04	1/21 4.7%	1/5039 0.0%
16	isocitrate dehydrogenase (NADP+) activity	4450	0.04	1/21 4.7%	1/5039 0.0%
17	long-chain-acyl-CoA dehydrogenase activity	4466	0.04	1/21 4.7%	1/5039 0.0%
18	2-methylcitrate dehydratase activity	47547	0.04	1/21 4.7%	1/5039 0.0%
19	glutathione peroxidase activity	4602	0.04	1/21 4.7%	1/5039 0.0%
20	oxidoreductase activity, acting on CH-OH group of donors	16614	0.04	4/21 19.0%	164/5039 3.2%

Discussion

This project aimed:

- To evaluate supplement usage
- To ascertain what benefits supplements may offer to phase 3 composts (in terms of mushroom yield and quality), and
- To add further understanding as to why/how supplements work

Compost supplements are in widespread usage throughout Europe and new products are entering the market. There has been no or little objective testing of these products previously.

The results of the trials show that the protein-based supplements in composts (at 1.5%) are beneficial for the mushroom industry while carboxylic acid based and mineral micronutrient based supplements in compost have no effect. Protein-based supplements in composts improve yields by an average of 11.5%, improve mushroom density by 5.5% (with knock-on effects on improved picking rates) and little or no effects on other quality characteristics such as flavour, colour and dry matter content. The value of the additional mushrooms produced by protein supplement use far exceeds the costs of the supplement. It is possible that further improvements to yield can be achieved by small increases in protein supplement rates above 1.5%, if compost temperatures can be controlled.

The final aim was to understand *how* supplements give benefits. As only two supplement types (protein and lipid/protein blend) were found to improve yields, the effects of these were examined by mycelial gene expression. The presence of lipids did change the physiology of the mycelium compared with non-supplemented and protein alone supplemented composts. This suggests that the lipids are stimulating yield by a different mechanism to the protein. Therefore, there are grounds to believe that further improvements in yield can be possible by more development work on the lipid/protein blends (e.g. formulation and composition).

Conclusions

Protein-based supplements have been shown to increase yield by an average of 11.5% on phase 3 composts. This finding was found for straw-based and horse manure-based composts. Any differences in yields between compost types (looking at Trials 2 and 3 separately) is interpreted as batch-to-batch variation and was possibly related to the moisture contents of the composts.

The two other supplement types tested, MycroNutrient (a carboxylic acid-based product) and Micromax (a mix of mineral micronutrients) had no effect on mushroom yields. MycroNutrient is marketed as a casing supplement, and it was tested here to investigate all possible supplement options. The negative result as a compost supplement does not contradict its claims as a casing supplement.

Micromax was tested because there are suggestions in the scientific and mushroom technology literature that mushroom compost may be deficient in minerals (particularly manganese) which can affect yielding (Royse and Beelman, 2008; Desrummaux, Calus and Sedeyn 2000). For some composts mineral nutrient deficiency may be a real problem but for composts used in these experiments, the input materials (straw, animal manures, gypsum, peat and lime) appear to have sufficient quantities of mineral micronutrients.

Supplement effects on mushrooms can be summarised as; no effects on flavour or dry matter content, protein supplements improve density/texture but cause a slight increase in mushroom yellow colour without affecting overall ‘whiteness’ (L value).

The increase in yield by protein-supplements is accompanied by an increase in density/texture. So there are three benefits of protein supplement usage: higher yields, improved texture and greater picking rates.

However, some evidence was found that two of the protein supplements (ProMycel Gold and McSubstradd) do result in slightly more ‘yellow’ mushrooms. It is not known whether these differences are noticeable to the consumer, since no differences were found between supplement use in the overall ‘whiteness’ of the mushrooms. The supplements Champfood E and the lipid/protein blend supplement (Natural Gold) did not cause mushroom yellowing but also did not affect overall whiteness.

The financial benefits of protein-supplement use are a 6.4 times multiplier: i.e. for every pound spent on protein supplement, £6.4 of extra mushrooms are produced. The limit of protein supplementation is set by the danger of high compost temperatures. Here, we have conducted experiments on a 50kg/tonne fill rate. Greater increases in yield are possible theoretically by increasing the supplement addition rate above 1.5%.

There may appear to be a contradiction between the findings that protein-supplements increase mushroom density/texture but do not change the dry matter contents. This is not so, as density relates to the density of cells in a mushroom, while dry matter content relates to how much of the composition of a cell is water or non-water. The fact that supplements have no effect on mushroom dry matter content would partially explain the fact that no flavour differences were found.

The gene expression analysis strongly suggests that the *A. bisporus* mycelium responds in a different way to the lipid/protein supplemented compost compared with non-supplemented compost or compost supplemented with protein alone. The presence of lipid as a nutrient appears to be detected by the mycelium stimulating a different metabolism. Lipids and their products have previously been shown to affect mycelial growth and mushroom production (Parker, 1993, Schisler and Patton, 1970). The 18-carbon lipid oil, linoleic acid, breaks down naturally to a 10-carbon compound, ODA (Oxa decanoic acid) and an 8-carbon compound, 1-octen-3-ol. Both of these compounds have been reported to have hormonal effects, ODA of cell growth and 1-octen-3-ol on mushroom pinning. Recent research suggests that 1-octen-3-ol is the key controlling factor for pinning with a greater controlling effect than CO_2 or temperature (Noble *et al*, 2009; Eastwood *et al.*, 2013).

However, despite the evidence that the supplementation with lipid/protein blends can stimulate a different metabolism in *A. bisporus* mycelium, the yield increase of the lipid/protein blend was the same as the protein alone. So at the moment there is no clear advantage in the use of lipid/protein blends, although the increased yellowing caused by protein-based supplements was avoided. This new information on the stimulation of a different metabolism may encourage further development onto the use of lipids as a partial supplement. The data from this project suggest that both protein and lipid/protein blend are acting as a direct nutrient source rather than a hormone as the dry weight of the increased yield is less than the weight of supplement: 15kg/tonne of supplement (or 13kg/tonne dry weight) yields an extra 42kg mushrooms which at 8% dry matter represents 3.36 kg extra dry weight.

It was a surprise to find only one gene differentially expressed in the compost mycelium between non-supplemented and protein supplemented composts. This could be due to the mycelium in non-supplemented compost having sufficient enzymic capacity to degrade and absorb the additional protein. In future it may be necessary to sample the compost more frequently to fully understand the physiology of mycelial breakdown of nutrient whether this is for chemical analysis, enzymic or gene expression studies. It was surprising that so many genes were differentially expressed between flushes 1 and 2. This may be an accurate result or somewhat influenced by time of sampling.

While no overall yield differences were found between the use of horse manure-based and straw-based composts, there were significant and consistent differences in quality attributes. Mushrooms grown on horse manure-based composts had a higher density while those grown on straw-based composts were whiter. The reasons for these differences are not known but they are likely to be chemically-based, and offer future opportunities for quality improvements.

The first trial showed that yield improvements of the brown strain, Heirloom, can be achieved using protein supplements.

In conclusion, the use of protein containing supplements (at 1.5%) in phase 3 compost was advantageous in raising the yields of mushrooms by an average of 11.5%. The increase in density by the use of protein-based supplements improve texture (but this was not detected by the Sensory Panel) and raise picking rates. The remaining quality attributes (mushroom colour, flavour and dry matter content) are changed to a small extent or not at all. Gene expression analysis revealed that the presence of supplemented lipids changed the metabolism of the mycelium but this was not reflected in any further increase in yield. This data provides sufficient evidence that further yield increases may be possible by small adjustments to the supplementation rates and development of lipid/protein supplements.

Knowledge and Technology Transfer

Progress on this project (M 56) has been presented to the HDC Mushroom Panel at meetings on 2 July 2013, 14 January 2014 and 2 July 2014, 9 October 2014 and 17 February 2015. A 20-minute presentation was made to mushroom growers at the Mushroom Technical Day, 18 March 2015 in Stratford-upon-Avon.

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Appendix

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food and drink innovation

Confidential report for:

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Report on: Sensory Evaluation of Mushrooms (Raw and Cooked) Using the Triangle Test Method (for difference) Determination of Effect of Nutritionally Supplementing the Mushroom Substrate on Mushroom Flavour

Report number: S/REP/133329/1 • Issue date: 25th June 2014

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SUMMARY

The mushrooms under test were grown in compost containing one of the following added nutritional supplements: Mycronutrient, Champ Food, Micromax and Natural Gold. In each case the test mushrooms (grown in the nutritionally supplemented compost) were compared against control mushrooms (grown in non-nutritionally supplemented compost) using the sensory Triangle Test Method (for difference) TES-S-001 and a panel of 18 sensory assessors (selected from the Trained Triangle Test Panel). The aim of the sensory testing was to determine whether the sensory taste panel could detect any overall sensory difference(s) between the two mushroom samples in each case (four separate tests on each of the four nutritional supplements). The mushrooms were evaluated as both raw and cooked mushrooms.

The overall aim of the research project was to understand mushroom nutrition; to improve mushroom yield, substrate efficiency and utilisation and flavour. The sensory element of the research focused on determination of whether the nutritionally supplemented composts had an effect on the test mushroom flavour when compared to control mushrooms grown in non-nutritionally supplemented compost.

Mycronutrient Nutritional Supplement:
Test Mushrooms (grown in Mycronutrient supplemented compost) compared against Control Mushrooms (grown in non-nutritionally supplemented compost).

Test Reference: 133329/1 Raw Mushrooms
For a triangle test for difference using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance. The test results show that 5 of the 18 assessors correctly identified the "odd" or "different" sample; we can therefore conclude that there is no statistically significant difference at the 5% alpha (α) significance level between the two mushroom samples.

Test Reference: 133329/8 Cooked Mushrooms
For a triangle test for difference using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance. The test results show that 9 of the 18 assessors correctly identified the "odd" or "different" sample; we can therefore conclude that there is no statistically significant difference at the 5% alpha (α) significance level between the two mushroom samples.

ChampFoods Nutritional Supplement:
Test Mushrooms (grown in ChampFoods supplemented compost) compared against Control Mushrooms (grown in non-nutritionally supplemented compost).

Raw Mushrooms Test Reference: 133329/2
For a triangle test for difference using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance. The test results show that 6 of the 18 assessors correctly identified the "odd" or "different" sample; we can therefore conclude that there is no significant difference at the 5% alpha (α) significance level between the two mushroom samples

Cooked Mushrooms Test Reference: 133329/7
For a triangle test for difference using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance. The test results show that 7 of the 18 assessors correctly identified the "odd" or "different" sample; we can therefore conclude that there is no significant difference at the 5% alpha (α) significance level between the two mushroom samples.

Micromax Nutritional Supplement:
Test Mushrooms (grown in Micromax supplemented compost) compared against Control Mushrooms (grown in non-nutritionally supplemented compost).

Raw Mushrooms Test Reference: 133329/3
For a triangle test for difference using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance. The test results show that 7 of the 18 assessors correctly identified the "odd" or "different" sample; we can therefore conclude that there is no significant difference at the 5% alpha (α) significance level between the two mushroom samples

Cooked Mushrooms Test Reference: 133329/6
For a triangle test for difference using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance. The test results show that 7 of the 18 assessors correctly identified the "odd" or "different" sample; we can therefore conclude that there is no significant difference at the 5% alpha (α) significance level between the two mushroom samples.

Natural Gold Nutritional Supplement:
Test Mushrooms (grown in Natural Gold supplemented compost) compared against Control Mushrooms (grown in non-nutritionally supplemented compost).

Raw Mushrooms Test Reference: 133329/4
For a triangle test for difference using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance. The test results show that 6 of the 18 assessors correctly identified the "odd" or "different" sample we can therefore conclude that there is no significant difference at the 5% alpha (α) significance level between the two mushroom samples

Cooked Mushrooms Test Reference: 133329/5
For a triangle test for difference using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance. The test results show that 5 of the 18 assessors correctly identified the "odd" or "different" sample we can therefore conclude that there is no significant difference at the 5% alpha (α) significance level between the two mushroom samples.

Note: Any opinions and interpretations contained in this report are outside the scope of UKAS accreditation and are highlighted in italics.

SAMPLE INFORMATION

Date samples received:	03/06/2014
Condition on receipt:	Good
Stored:	As requested by the client all mushroom samples were stored under refrigerated conditions in the Sensory Walk in Chiller (room 908) prior to testing
Date samples tested:	Samples tested raw – 04/06/2014 Samples tested cooked – 05/06/2014

SAMPLE DESCRIPTION

Campden BRI Code	Company Code/Sample Description
S/133329/1	Control Mushrooms - Non supplemented compost
S/133329/2	Test Mushrooms - Mycronutrient supplemented compost
S/133329/3	Test Mushrooms - ChampFood supplemented compost
S/133329/4	Test Mushrooms - Micromax supplemented compost
S/133329/5	Test Mushrooms - Natural Gold supplemented compost

METHODS AND REFERENCES

Method reference:	Triangle Test No. TES-S-001 (British Standard, Sensory Analysis -Methodology - Triangle Test BS EN ISO 4120: 2007)
Deviations from method:	None

AIM

The aim of the sensory element of the research focused on determination of whether the four nutritionally supplemented composts: Mycronutrient, Champ Food, Micromax and Natural Gold had an effect on the test mushroom flavour when compared to control mushrooms grown in non-nutritionally supplemented compost. The mushrooms were tested using the sensory Triangle Test Method (for difference) and a panel of 18 sensory assessors (selected from the Trained Triangle Test Panel). Tests were conducted on both raw and cooked mushrooms.

PREPARATION

Raw Mushrooms

The mushrooms were gently wiped clean using dry paper towel, stalk ends removed and the mushrooms cut into quarters and placed into two separate coded glass bowls. The samples were then transferred into coded sample containers following the experimental design of the test. Each assessor received two quarter pieces of mushroom per coded container.

Cooked Mushrooms

The mushrooms were gently wiped clean using dry paper towel and the stalk ends removed. Several baking trays (one tray per mushroom sample variant) were each covered in aluminium foil. Approximately 10ml of vegetable oil was added to each tray and the prepared mushrooms added and mixed until lightly coated in the oil. The trays of mushrooms were placed into a pre-heated fan oven for approximately 10 minutes at 170°C until fully cooked. Once cooked each mushroom was cut into quarters and placed into a pre-heated coded Bain Marie pan to maintain the temperature of the samples throughout the test. To ensure the sensory quality of the mushroom samples was not compromised a second batch of mushrooms was prepared half way during the panel session. The mushroom samples were served warm (presented as the assessors arrived at the sensory booths) and presented following the experimental design of the test. Each assessor received two quarter pieces of cooked mushroom per coded container.

SENSORY TESTING

The samples were evaluated using the Triangle Test Procedure (TES-S-001). In the triangle test assessors are presented with a set of three coded samples, two of which are the same and one of which is different. The sets of samples are presented equally often in each of the six possible orders; this experimental design minimises any possible order and carryover effects.

Eighteen trained assessors are used for each test, nine receiving "test" as the different sample and nine receiving "control" as the different sample. After tasting the three samples in the designated order, each assessor is asked to select the "different" sample and to describe the difference(s) perceived.

TEST CONDITIONS

The test was carried out in a purpose-built testing room. Each assessor was required to undertake the tests in an individual booth. The room was positively pressurised to minimise the entrance of external odours. Coloured lighting (red) was used to mask any colour difference between the samples. The panel used water crackers and filtered water as palate cleansers between the samples to minimise sample carry-over.

TRIANGLE TEST RESULTS

Table 1: Results of Triangle Test - Mycronutrient

Test Reference No.	No. of Assessors	No. Correctly Identifying the Different Sample	Significance
133329/1 Raw Mushrooms Control versus Test (Test mushrooms grown in Mycronutrient supplemented compost)	18	5	NSD at the 5% alpha (α) level of significance
133329/8 Cooked Mushrooms Control versus Test (Test mushrooms grown in Mycronutrient supplemented compost)	18	9	NSD at the 5% alpha (α) level of significance

For a triangle test for difference using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance.
Reference: Sensory Analysis Methodology – Triangle Test BS EN ISO 4120: 2007

NSD – No Significant Difference

Descriptors given when the different sample was correctly identified can be seen in Table 2.

Table 2: Descriptors - Mycronutrient

Test Reference No: 133099/1 – Raw Mushrooms

S/133329/1 Control Raw Mushrooms	Stronger flavour (1) Weaker taste (1) Earthy (1)
S/133329/2 Test Raw Mushrooms (Test mushrooms grown in Mycronutrient supplemented compost)	Weaker flavour (1) Sweeter than the other two samples (1)

Test Reference No: 133099/8 – Cooked Mushrooms

S/133329/1 Control Cooked Mushrooms	More bitter (1) Slight metallic taste (1) Fresher taste (1) Much softer texture and stronger perfumed note (1) Very little difference between the samples, this sample was juicier and stronger in flavour (1) No difference (1)
S/133329/2 Test Cooked Mushrooms (Test mushrooms grown in Mycronutrient supplemented compost)	Bitter and metallic taste (1) More bland, less sharp aftertaste (1) Slightly sour aftertaste, less sweet (1)

() Number of assessors using the descriptor

TRIANGLE TEST RESULTS

Table 3: Results of Triangle Test - ChampFood

Test Reference No.	No. of Assessors	No. Correctly Identifying the Different Sample	Significance
133329/2 Raw Mushrooms Control versus Test (Test mushrooms grown in ChampFood supplemented compost)	18	6	NSD at the 5% alpha (α) level of significance
133329/7 Cooked Mushrooms Control versus Test (Test mushrooms grown in ChampFood supplemented compost)	18	7	NSD at the 5% alpha (α) level of significance

For a triangle test for difference using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance. Reference: Sensory Analysis Methodology – Triangle Test BS EN ISO 4120: 2007

NSD – No Significant Difference

Descriptors given when the different sample was correctly identified can be seen in Table 4.

Table 4: Descriptors - ChampFood

Test Reference No: 133099/2 – Raw Mushrooms

S/133329/1 Control Raw Mushrooms	Milder flavour (1) Stronger taste (1) Drier, earthy taste (1) Guess/ no difference (1)
S/133329/3 Test Raw Mushrooms (Test mushrooms grown in ChampFood supplemented compost)	Fresher, cleaner taste (1) Guess/ no difference (1)

Test Reference No: 133099/7 – Cooked Mushrooms

S/133329/1 Control Cooked Mushrooms	Stronger savoury mushroom taste (1) More savoury (1) Weaker, harsh, other two samples slightly sweet (1) Sharper taste, stronger aftertaste (1) Sour aftertaste, earthy (1)
S/133329/3 Test Cooked Mushrooms (Test mushrooms grown in ChampFood supplemented compost)	Salty and strong in flavour, other two samples were very earthy (1) Slightly greasy taste (1)

() Number of assessors using the descriptor

TRIANGLE TEST RESULTS

Table 5: Results of Triangle Test - Micromax

Test Reference No.	No. of Assessors	No. Correctly Identifying the Different Sample	Significance
133329/3 Raw Mushrooms Control versus Test (Test mushrooms grown in Micromax supplemented compost)	18	7	NSD at the 5% alpha (α) level of significance
133329/6 Cooked Mushrooms Control versus Test (Test mushrooms grown in Micromax supplemented compost)	18	7	NSD at the 5% alpha (α) level of significance

For a triangle test for difference using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance.
Reference: Sensory Analysis Methodology – Triangle Test BS EN ISO 4120: 2007

NSD – No Significant Difference

Descriptors given when the different sample was correctly identified can be seen in Table 6.

Table 6: Descriptors - Micromax

Test Reference No: 133099/3 – Raw Mushrooms

S/133329/1 Control Raw Mushrooms	Odd, strange taste? Soapy perhaps? (1) Bland taste (1) No difference – guess only (1)
S/133329/4 Test Raw Mushrooms (Test mushrooms grown in Micromax supplemented compost)	Overall less flavour and less perfumed notes (1) Slightly more waxy mouthfeel and sweeter taste (1) Earthy raw flavour (1) No difference (1)

Test Reference No: 133099/6 – Cooked Mushrooms

S/133329/1 Control Cooked Mushrooms	More flavour, saltier, umami flavour (1) More oily; "thicker " taste (1) Metallic and bitter taste (1) More watery, metallic taste (1) Less sweet (1)
S/133329/4 Test Cooked Mushrooms (Test mushrooms grown in Micromax supplemented compost)	"Better" fresher taste (1) Difference in texture (1)

() Number of assessors using the descriptor

TRIANGLE TEST RESULTS

Table 7: Results of Triangle Test

Test Reference No.	No. of Assessors	No. Correctly Identifying the Different Sample	Significance
133329/4 Raw Mushrooms Control versus Test (Test mushrooms grown in Natural Gold supplemented compost)	18	6	NSD at the 5% alpha (α) level of significance
133329/5 Cooked Mushrooms Control versus Test (Test mushrooms grown in Natural Gold supplemented compost)	18	5	NSD at the 5% alpha (α) level of significance

For a triangle test for difference using 18 assessors, a minimum number of 10 correct responses are required to establish a significant difference between samples at the 5% alpha (α) level of significance.
Reference: Sensory Analysis Methodology – Triangle Test BS EN ISO 4120: 2007

NSD – No Significant Difference

Descriptors given when the different sample was correctly identified can be seen in Table 8.

Table 8: Descriptors

Test Reference No: 133099/4 – Raw Mushrooms

S/133329/1 Control Raw Mushrooms	Stronger flavour (1) Sweeter (1) Much softer texture, other two samples were firmer and slightly crumbly (1) No difference/guess (2)
S/133329/5 Test Raw Mushrooms (Test mushrooms grown in Natural Gold supplemented compost)	Bland flavour and rubbery texture (1)

Raw Test Reference No: 133099/5- - Cooked Mushrooms

S/133329/1 Control Cooked Mushrooms	Less sweet and less "fresh" taste (1) Stale, bitter (1) Very slightly stronger flavour but no real difference (1)
S/133329/5 Test Cooked Mushrooms (Test mushrooms grown in Natural Gold supplemented compost)	Slightly saltier, more flavour (1) Weaker aftertaste (1)

() Number of assessors using the descriptor

