

**Substantial Equivalence Application for the
Approval of a Lentinan-rich extract, Lentinex®,
derived from the Mushroom, *Lentinus edodes*,
as a dietary supplement**

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Non-confidential



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INTRODUCTION

Lentinex[®] is an aqueous extract of the Shiitake mushroom (*Lentinus edodes*) and is manufactured by MediMush AS, a Danish food company. It is a clear, light brown liquid containing glucose, other sugars, protein and β -glucans. All of these compound classes have already received GRAS status. The Shiitake mushroom itself has been marketed extensively in the EU prior to 1997 while aqueous extracts of the mushroom have also been marketed either as capsules or liquid extracts prior to 1997. MediMush intend to market Lentinex[®] for use as a dietary supplement companies within the EU as an alternative form of the extracted β -glucans from the Shiitake mushroom. This will present a standardised β -glucan product that is free from fungal cellular debris, has a long shelf life and offers consumers an increased choice when selecting β -glucan containing products.

Approval of this product is being requested under EC Regulation No. 258/97 which is conceived with the introduction of novel foods and ingredients into the EU and ensures that the novel food in question has been assessed for its safety prior to its introduction to the general public. For plant (mushroom) derived foods that have a safe history of use a simplified procedure for pre-market approval can be utilised providing that the food product can be demonstrated to be substantially equivalent with regard to the composition, nutritional effects, and potential toxicity as outlined in Article 3.4 of the Regulation 258/97. According to the procedure laid down in Article 3.4, the present submission will put forward the case that Lentinex[®], an aqueous extract of fermenter-produced mycelium of the Shiitake mushroom, when used in capsules or aqueous extracts, is substantially equivalent to an existing Shiitake derived β -glucan, Bio-Life, currently on sale and was on sale prior to 1997 within the EU. Furthermore, since Lentinex[®] is derived from plant (mushroom) material

obtained from non-GM sources, the classification under section 4, “*Scientific Classification of Novel Foods for the Assessment of Wholesomeness*” which facilitates the nutritional and safety of the novel foods, is applicable. Lentinex[®] is classified as class 2 “*Complex NF from non-GM Sources*” and is also applicable to sub-heading 2.1 “*The source of the NF has a history of food safety*”.

1.0 HISTORY OF USE OF THE MUSHROOM *LENTINUS EDODES* AS A SOURCE OF THE β -GLUCAN – LENTINAN

Lentinus edodes, taxonomic classification: *Lentinus edodes* (Berk.) Sing. (Agaricomycetidae) is a mushroom fungus (known widely as the Shiitake mushroom) indigenous to Japan, China and other Asian countries with temperate climates and is usually found growing on fallen deciduous trees (Bensky and Gamble, 1993). However, it has been grown artificially for several centuries on cut logs and, more recently, worldwide mass cultivation is predominantly achieved by enriched sawdust culture technology (Stamets, 2000; Chang and Miles, 2004). Fresh Shiitake mushrooms are now produced widely in the UK and Europe, and are available in most supermarkets and fresh food stores. In the USA, the fresh mushroom is approaching the sales level of the common white button mushroom. In Japan and China it has long been a regular part of dietary intake in fresh and dried form.

In China it is known as Xiang gu (fragrant mushroom) and as Shiitake in Japan because of the historical association with the Shia tree. This mushroom has been renowned in Japan and China for thousands of years both as a food and as a component of Traditional Chinese Medicine (TCM). Furthermore, the exotic and delicious taste of this mushroom is a central part of many Oriental dishes which are increasingly being adopted in the West mainly due to the greatly increased availability of Shiitake mushrooms (Carluccio, 2003). In the UK, the major growers of fresh Shiitake are in Lancashire and Callander (Scotland).

Shiitake is now established as the leading mushroom worldwide that can be used both as a nutritious and tasty food, and as a rich source of β -glucans. The natural growing process utilises complex, lignocellulytic substrates of undefined chemical composition. Furthermore, the fungus can also be grown in a mycelial form

in controlled, nutrient defined liquid fermentation (bioreactor) and it is from this source that Lentinex[®] is prepared, and is the subject of this application. By means of batch, liquid fermentation, a pure, uniform, repeatable mass of biomass can be generated with a more easily defined repeatable organic composition.

Lentinus edodes is a source of a well-studied cell wall β -glucan or polysaccharide – Lentinan – which can be extracted from both the fruit-body (the mushroom), from the liquid cultivated mycelium and also extruded into the fermentation broth (Ohno, 2005). In dietary supplement form the β -glucans are present in a much higher concentration than can be achieved realistically by consuming fresh mushrooms.

Commercial dietary supplement concentrates from several mushrooms are available as tablets, capsules or elixirs and are widely on sale in most Oriental countries, and in the USA and increasingly in Europe in natural food/health stores. Such dietary supplement sales worldwide have been estimated at between 1.5-2 billion US dollars annually.

2. ORIGIN AND OCCURRENCE OF β -GLUCANS INCLUDING LENTINAN IN NATURE

β -glucans are a structurally diverse group of biological macromolecules of widespread occurrence in nature and have been part of the food chain for millennia. A recent study showed that Lentinan and other β -glucans are present in a number of common foods. Six food categories, including legumes, cereals, tubers, vegetables, fruits and mushrooms have been shown to contain Lentinan. Analysis of the food fractions showed that besides the well-known Lentinan-containing *Lentinus edodes* mushroom popular foods such as celery, chin-chian leaves, carrot and radish contained nearly 20% β 1,3 -glucan in their total carbohydrate fraction. Soybean dry weight contained 0.8%, β 1.3 -glucan which was twice the amount compared to Shiitake mushrooms (see Table 1). However, the major source of β -glucans including Lentinan is in mushrooms.

β -glucans are composed of repetitive structural features that are polymers of monosaccharide residues joined to each other by glucosidic linkages. In this way they differ structurally from proteins and nucleic acids. Polysaccharides present the highest capacity for carrying biological information since they have the greatest potential for structural variability. The amino acids in proteins and the nucleotides in nucleic acids can interconnect in only one way while the monosaccharides can interconnect at several points to form a wide variety of branches or linear structures (Sharon and Lis, 1993).

Polysaccharides isolated from mushrooms in general (fruit-body, submerged cultured mycelial biomass, or liquid culture broth) are either water soluble β -D-glucans, β -D-glucans with heterosaccharide chains of xylose, mannose, galactose and uronic acid or β -D-glucan protein complexes – proteoglycans (Mizuno, 1999).

All of these polysaccharide forms have been shown to offer health benefits in man.

Lentinan is a water-soluble β -D-glucan derived from *Lentinus edodes*.

Table 1: Foods containing β -glucans (Ko and Lin, 2004)

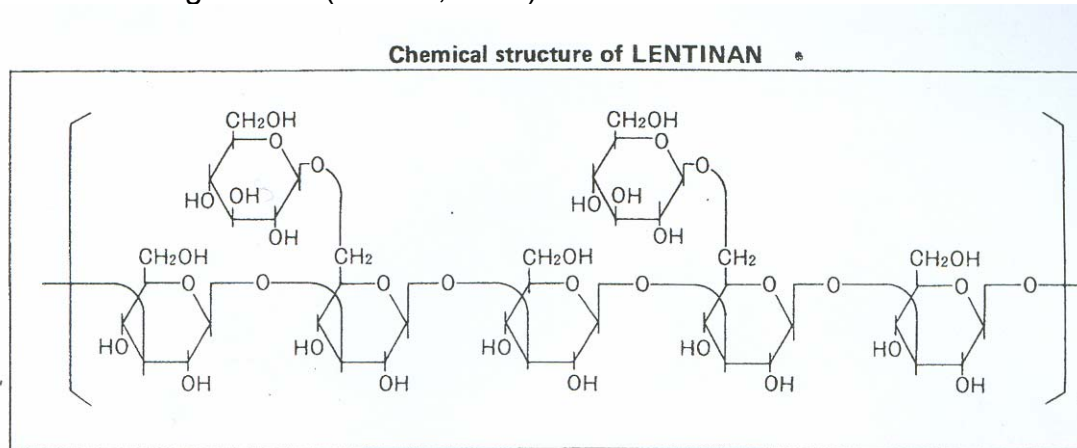
1,3 - β - Glucan Content of the Extracted Fractions in Food	(Dry Weight)	
	TOTAL	Water %
Soybean	0.82±0.37	10.0
mung bean	0.37±0.10	9.05
Indian Bean	0.16±0.01	9.0
Adlay seeds	0.38±0.09	4.5
Oat	0.41±0.12	9.0
Chinese Cabbage	0.15±0.03	95.0
Celery	0.24±0.02	94.1
Chin-chian	0.53±0.01	N/A
-	0.27±0.04	88.2
Radish	0.39±0.00	94.5
Taro	0.28±0.21	95.0
Banana	0.35±0.02	75.7
Apple	0.22±0.00	84.4
Pear	0.21±0.01	83.2
Shiitake Pileus	0.37±0.01	90.0
Snow Mushroom	2.54±0.52	92.0
Juda's Ear	0.28±0.25	90.0

2.1 Chemistry of Lentinan

Lentinan (IUPAC) is a β -(1-3) β -(1-6)-D glucan and has a molecular weight of approximately 5×10^5 Daltons, the degree of branching is 2/5 and the tertiary structure of Lentinan is a triple helix (Fig. 1). Lentinan's 2006 Medical Subject Headings Descriptor Data are: Tree: DO9.698.365.089.500 (National Library of Medicine) and its Registry Number is 9051-97.

In contrast, β -glucans from oats, and currently used extensively as dietary supplements, are comprised of (1-3),(1-4) β -D-glucan linkages.

Fig. 1. Lentinan is a β -1,3-glucan with a high molecular mass. Its fundamental structure consists of a unit of two β -(1-6) branches per five glucose residues of straight chain (Mizuno, 1999).



Lentinan is a high molecular weight polysaccharide in a triple helix structure containing only glucose molecules with mostly β -(1-3)-glucose linkages in the regularly branched backbone, and β -(1-6)-glucose side chains. The configuration of the glucose molecules in a helix structure is considered to be important for the biological activity (Fig. 1). Lentinan is completely free of any nitrogen (and thus protein), phosphorus, sulphur and any other atoms except carbon, oxygen and hydrogen. It is water soluble, heat stable and alkali labile.

3. CURRENT METHODS FOR PRODUCING DIETARY SUPPLEMENTS (DSs) FROM MUSHROOMS SUCH AS *LENTINUS EDODES*

There are presently several approaches for producing DSs from *L. edodes* and other mushrooms (Wasser, 2005):

1. Dried and pulverised naturally growing mushroom fruit-bodies in the form of capsules or tablets.
2. Artificially cultivated fruit-bodies dried and pulverised, hot water or alcohol extracts from them, or the same extracts concentrated and their mixtures (e.g. Bio-Life).
3. Dried and pulverised preparations of the combined substrate, mycelium and mushroom primordia following inoculation of edible semi-solid medium (usually grains).
4. Biomass or extracts of mycelium or the broth harvested from submerged liquid cultures grown in bioreactors (e.g. PSK and PSP from *Trametes versicolor*, Schizophyllan from *Schizophyllum commune* and Lentinex[®] from *Lentinus edodes*).

There are currently no standard protocols for guaranteeing edible mushroom DSs for product quality and efficacy. Many mushrooms, including *Lentinus edodes*, have been used for traditional health benefits for long periods of time, in some cases for thousands of years. There are few documented examples of adverse effects to man and as such could be considered as 'safe'. However, from a pharmacological point of view, safety is a relative concept and it is clear that safety of all mushroom-derived DSs cannot be guaranteed just simply because they have mostly many centuries of use.

Reliance on traditional use as an indication of safety involves a danger, namely, the poor information available to us from antiquity. It is now clear that safety criteria for edible mushroom preparations should be based solely on modern scientific evidence and not to rely on inadequate historical evidence. Furthermore, it is reassuring that when compared with many herbal preparations, mushroom preparations show little evidence of overt toxicity. The main advantage of using mushroom-based DSs with respect to safety (as compared to herbal preparations) include the following:

1. The overwhelming majority of mushrooms such as *Lentinus edodes* used for the production of DSs are cultivated artificially (and not gathered in the wild) and, consequently, guaranteeing proper identification and pure and unadulterated products. Proper culture maintenance ensures genetic uniformity and stability.
2. Mushrooms are easily propagated vegetatively and thus keep to one clone. The mycelium can be stored for long periods of time and the genetic and biochemical consistency may be regularly checked.
3. Undoubtedly the most important modern advantage is the fact that many mushrooms, such as *Lentinus edodes*, are capable of growing in the form of mycelial biomass in submerged controlled fermenter (bioreactor) culture (Lentinex[®]). *Particularly, from a safety aspect, this will undoubtedly be the major forward direction for many aspects of mushroom DSs* (Wasser *et al.*, 2000).

Marketed DSs from *Lentinus edodes* are currently mostly being derived from whole fresh/or dried mushrooms. Such products normally give no indication of the exact amount, if at all, of Lentinan or other polysaccharides present.

The task of converting the very diverse raw materials of *Lentinus edodes* into a consistent product will reflect industrial practices and standards and on methods of assessing efficacy. Whole mushrooms are complex structures both morphologically and physiologically with undoubted variations in chemical composition from batch to batch. The composition of a mushroom fruiting body will reflect substrate composition and ingredients which can vary considerably since the basic raw materials are normally of agricultural or forestry origin. Thus, the current use of the complex mushroom fruit-body does imply that the standardisation of the DS from medicinal mushrooms is problematic.

The use of pure culture mycelial cultivation in defined liquid fermentation conditions will offer several advantages over traditional whole mushroom methods (Wasser *et al.*, 2004):

1. Speed of growth with reduction in production time;
2. Optimisation of culture medium composition;
3. Optimisation of physico-chemical conditions to allow regulation of mushroom metabolism; and
4. Improved yield of specific products.

Increasingly in other parts of the world (such as Japan), producers of DSs such as Lentinan are moving or have already moved over to fermenter produced products. Clearly, this will increasingly become the mode of choice for production of many mushroom DSs. *Such fermenter methods will give increased levels of reproducibility and known concentration and consistency of the main products. Lentinex[®] is an example of such methods.*

4. DESCRIPTION OF MEDIMUSH PROCESS FOR PRODUCING LENTINEX® (CONFIDENTIAL)

MediMush has developed a sophisticated production technology based on the cultivation of several different medicinal mushrooms in aseptic submerged liquid cultivation (1,500 litre vessel) which allows the manufacture of ingredients with purity above 98%. This method uses stable pure cultures of the fungus, known measured medium ingredients, and carefully arrived at environmental conditions, e.g. temperature, aeration rate etc. This method can be utilised to manufacture different β -glucans derived from different mushroom species.

The MediMush process cultivates *L. edodes* mycelium in a liquid fermentation process that ensures complete control of the growth and physiology of the organism ensuring a constant supply of a clear, product of defined composition (Tables 2-3).

Table 2: Specifications for Food Grade Material: Lentinex®

Main Ingredients

Total sugars	13 mg/ml
(free glucose)	12 mg/ml
Protein	0.4 mg/ml
β -glucan (Lentinan)	0.5 mg/ml

Table 3: Lentinex® has minimal or undetectable levels of heavy metals and pesticides

Heavy metals

Cd	< 0,01 mg/kg
Cr	0,15 mg/kg
Mu	0,11 mg/kg
Hg	< 0,02 mg/kg
Pb	< 0,02 mg/kg
Pesticides	No pesticides present

The production is carefully maintained and Quality Assurance programmes have been established. The entire process is under the control and management of Dr Bjorn Kristiansen, an internationally recognised microbial biochemical engineer, who formally held the Chair in Bioprocess Technology at the University of Strathclyde, Glasgow, Scotland.

There are now numerous examples of *Lentinus* type powders and extracts within the European market. In food processing, bulk quantities of Shiitake powders derived for the Far East are added into various sauces and other condiments while in the health food market there are numerous forms of *Lentinus edodes* products, capsules or elixirs, now available.

For the purpose of this substantial equivalence presentation one product from Bio-Life was selected for comparison with Lentinex®. The product is marketed by:

Bio-Life Laboratorial Natural Products
Parc Scientifique CREALYS
Rue Camille Hubert 33
B-5032 ISNES
BELGIUM

The Bio-Life product is in the form of tablets based on dried pulverised fruit-bodies of *Lentinus edodes*, and has been in the European market since the 1990s.

5. CASE FOR SUBSTANTIAL EQUIVALENCE OF LENTINEX® WITH BIO-LIFE

5.1 Composition

Mushroom products are sold as dried mushroom powders or extracts. Bio-Life, the product that is used as a comparison with Lentinex®, is sold as a dried powder of *Lentinus edodes* fruit-bodies. To determine the content of the powder, the method universally employed is to immerse the powder in boiling water for a predetermined period and to remove the debris by filtration. The resulting liquor is taken to represent the bioavailable content of the mushroom powder.

In the case of Lentinex®, the biomass is removed from the fermentation broth by filtration and the presence of active ingredients is determined in the remaining liquor by suitable analytical techniques.

Thus, the comparison between Bio-Life and Lentinex® is based on the content of two similar liquids.

Fresh mushrooms contain more than 80% water. Removing the water, by for example drying, yields a powder where the concentration of ingredients of interest will be much higher than in the fresh mushroom. Thus, mushroom products in the form of powders or extracts may be regarded as a mushroom concentrate, as indeed is Lentinex®.

The reason for buying mushroom products is that they contain compounds that are claimed to be good for your health. The most important of these ingredients are polysaccharides. In the case of Shiitake, the best-known ingredient is Lentinan, a beta-glucan with a molecular weight of above 500 kDa, which is also found in other food materials such as yeasts, oats, etc. Lentinan is claimed to be the main reason as to why the Shiitake mushroom is so popular.

5.1.1 Determination of polysaccharide content

The standard method for determining the level of polysaccharides is precipitation with a suitable solvent and weighing the precipitate after drying. However, the problem with this method is that other compounds, such as proteins, will also precipitate out. This may not be a problem for a fast determination of overall polysaccharide content, as the protein content of these products is very low, but it does not provide any insight into the composition of the polysaccharides. For both BioLife and Lentinex®, the level of polysaccharides as determined by precipitation with absolute ethanol is 0.3 - 1 mg/ml. To find out the individual sugar component of the polysaccharide it is necessary to break it down using acid hydrolysis and determine the presence and level of individual sugars using HPLC.

For both Bio-Life and Lentinex® it was found that the polysaccharides were composed of three sugars only, namely glucose as the main ingredient and smaller amounts of galactose and mannose. The sugar composition is given in Table 5 where the presented data is given as molar ratios and normalised with respect to glucose. Thus Lentinex® will contain 0.3 mole of galactose and 0.1 mole of mannose for each mole of glucose.

Table 4. Sugar composition of the polysaccharides in Bio-Life and Lentinex®

Product	Glucose	Galactose	Mannose
Bio-Life	1	0.1	0.1
Lentinex®	1	0.1	0.3

According to Table 4, the polysaccharides in both products are composed of the same sugars and what is even more interesting is that the compositions are nearly identical. The polysaccharides in Lentinex® have slightly more mannose than in Bio-Life, but this will be of no significance nutritionally. The mannose comes from the ingredients in the growth medium.

To determine the content of the fraction of the polysaccharides that contains the Lentinan it is necessary to separate the low molecular weight fraction from the high molecular weight fraction with the latter being the fraction of interest. The sugar composition of the polysaccharides with a molecular weight above 500 kDa is given in Table 5 where the data is presented as molar ratios normalised with respect to glucose content as in Table 4.

Table 5 Sugar composition of the polysaccharides with a molecular weight above 500 kDa in Bio-Life and Lentinex®

Product	Glucose	Galactose	Mannose
Bio-Life	1	0.1	0.1
Lentinex®	1	0.1	0.2

Again, it can be seen that the products contain identical sugar monomers and in this case their composition is identical.

As these mushroom products will be consumed primarily for their polysaccharide content, it can be argued that they are approximately identical. However, there may be dissimilarities in terms of the level of other components, such as protein and presence of contaminating ingredients such as heavy metals and pesticides.

5.1.2 Determination of protein

The levels of protein found in the Bio-Life extract and Lentinex® is given in Table 6.

Table 6 Protein level in Bio-Life and Lentinex®

Product	Protein (mg/ml)
Bio-Life	0.05
Lentinex®	0.04

It can be seen that the protein level in Bio-Life is higher than in Lentinex®, but the levels are very low in both cases, compared to the levels of polysaccharides.

5.1.3 Fats

The fat content in both Bio-Life and Lentinex® is negligible. In the case of Lentinex®, it cannot be detected.

For both these products, the composition of the main ingredient, the polysaccharides, and the level of other major constituents are very similar. This indicates that a claim of substantial equivalence is justified on compositional grounds.

5.2 **Nutrition**

The shiitake mushroom can occasionally produce variable amounts of vitamin, terpenes and alkaloids, depending on the growth conditions. However, the levels will be insignificant in both products and are expected to have no impact on the nutritional value of the products. Therefore, both the Bio-Life product and Lentinex® are vehicles for supplying polysaccharides and small amounts of protein. The polysaccharides are nearly identical and, hence, provide very similar nutritional values.

5.3 Metabolism

It has not been possible to obtain any relevant information on the influence of the Bio-Life product on animal and human metabolism.

In contrast, Lentinex® has been extensively studied with several animal species, *viz.* mouse, rat and pig (see Appendix 1) and there is sound evidence that Lentinex® does not cause any significant or harmful changes in metabolism of these species, under the intended conditions of planned use. These results are relatively in agreement with Far East studies on the safety of Lentinan (Appendix 2).

A cross-over placebo, controlled human study with Lentinex® again demonstrated the safety of this product in a group of healthy elderly humans (Appendix 3).

5.4 Intended Use

Lentinex is intended for use as a food supplement, or dietary supplement, similar to the Bio-Life product. The term dietary supplement was formally defined by the US administration in 1994 as a product intended to supplement the diet to enhance health (Dietary Supplement Health and Education Act, Public Law 103-417, 1994.). Lentinex is intended for ingestion in the form of a capsule (akin to Bio-Life), a liquid, powder, gel, etc.

5.5 Level of Undesirable Substances

The presence of pesticides and heavy metals are well known in traditionally grown mushroom products (Chang and Miles, 2004). However, the levels vary with growth season and location of production, harvesting procedures and method of cultivation. Many European suppliers of mushroom products are aware of this and endeavour to sell good quality products. However, this is not always the case. The

conditions for the production of the mushroom fruit-bodies used in Bio-Life production are not known.

Lentinex® is manufactured using drinking water and substrates that are approved for food use. Therefore, Lentinex will not contain heavy metals and pesticides at levels above the level found in drinking water (see Table 3).

5.6 Stability of Lentinex®

There is no published information of stability of β -glucan based products from mushrooms. Since traditional mushroom products such as Bio-Life powders are generally regarded as stable products, MediMush undertook stability studies on Lentinex®. The stability was evaluated in terms of the polysaccharides present in the liquid. In an unstable product, the polysaccharides will be broken down into smaller fragments and finally to monomeric sugar molecules. Thus, for the evaluation of any breakdown, the relative viscosity and the concentration of polysaccharide can be determined. As Lentinex® contains large molecules, a falling relative viscosity will indicate a breakdown of the larger molecules into smaller fractions, To determine the concentration of the polysaccharide, precipitation with absolute ethanol was used as described earlier in this document. The results are given in Table 7. Note that the table contains relative values, all normalised with regard to the initial values measured at the end of the relevant production batch. Thus a figure of 1.0 indicates no change, i.e. no breakdown observed.

Table 7. Stability test for Lentinex®

Storage time	1 month	4 month	12 months
Heated to 80 °C and stored at 4 °C	Conc: 1.0	Conc: 0.96	Nd
No heat treatment and stored at 4 °C	Conc 1.0	Conc 1.0	Nd
No heat treatment and stored at 20 °C	Viscosity: 1.0 Conc: 1.0	Viscosity: 1.0 Conc: 1.0	Viscosity: 1.0 Conc: 1.0

Appendix 1

Safety and Pharmacological Effects of Lentinex®

In an initial toxicity and efficacy study, Lentinex® was administered to 10 BN rats via oral lavage, 2.5 mL and 3.1 mL daily in 5 day cycles, with a 2 day rest between cycles. All rats were observed for weight loss, lethargy and for toxic effects, ataxia and behavioural changes. Initially (Wk 7), rats were administered 2.5 mg/kg for 3 cycles. This dose of Lentinex® was tolerated well with no ill-effects observed and regular weight gain as observed with untreated controls. However, after 3 cycles of treatment there was no observed change in any haematological component from basal levels. Thus, the dose of Lentinex® was increased to 5 mg/kg (Wk 10). Again, animals displayed no toxic effects and continued to gain weight at the same rate as the untreated controls.

While after the initial period of administration (Wks 7-10) with 2.5 mL (0.5 mg/ml) of Lentinex®, there were no observed changes in haematological components, following administration of the first cycle of 3.1 mL (0.8 mg/ml) per day there was an observed increase in average platelet production ($P > 0.05$, Wk 11) from basal levels which increased further ($P < 0.05$) following 2 additional cycles (Wk 13). Subsequent to a total of 9 cycles of this dose of Lentinex®, average platelet levels have declined from these initially high values. Concurrently, average red blood cell counts (RBC) increased from basal levels by Wk 13 ($P < 0.05$) and remained higher than basal levels up to Wk 19 ($P < 0.05$), with a slight but statistically insignificant increase in hemoglobin levels by Wk 13 ($P > 0.05$). White blood cells counts (WBC) decreased initially from basal levels by Wk 11 ($P < 0.05$). However, increased levels were observed on Wk 13 in comparison to Wk 11 ($P < 0.0001$) and basal levels (Wk 9; $P > 0.05$) with levels dropping well below basal levels by Wk 19 ($P < 0.0001$). While percentages of CD8 and CD4 positive cells remained relatively unchanged there was

a considerable increase in both the B-cell ($P < 0.0001$) and monocyte populations ($P < 0.0001$) on Wk 13, consistent with the above observations of increases on Wk 13.

Interferon gamma increased significantly in comparison to controls by Wk 11 ($P < 0.01$), with an apparent, consistent escalation following increasing length of Lentinex® administration. There were no significant alterations of tumour necrosis factor alpha, IL-1, IL-1 or IL-6. IL-2 exhibited a transient decrease at week 11. Following an initial increase in IL-10 by Wk 9, levels subsequently normalized up to and including Wk 19. GM-CSF remained unchanged until Wk 19 when levels increased, but with a spread of data points.

MediMush concluded “Animals have displayed no physiological toxic side-effects to Lentinex® at the doses described and continue to thrive following 34 weeks of administration. There are no indications of haematological toxicity on the subset of blood cells or parts of the immune system examined following Lentinex® administration.”

In a series of two rat studies Lentinex® was administered at 2,5-5 mg/kg via oral lavage daily to rats that were previously injected with BNML leukemia. All rats were observed for weight loss, lethargy and hind limbs were monitored for paralyses. In addition, the Lentinex® group was monitored for toxic effects, ataxia and behavioral changes. While no adverse events were noted for any of the Lentinex® treated BNML rats, neither did the Lentinex increase survival of these rats compared to controls. It was concluded that while Lentinex® was safe at these doses, the doses were not high enough to get a therapeutic effect.

Following these studies, two additional studies were conducted. In the first study Lentinex® was administered at 0.5 mg/kg, and Idarubicin (1.5 mg/kg) plus Lentinex® at 0.5 mg/kg were administered orally to rats that were then later injected

with BNML leukemia. In the second study Lentinox® at 20 mg/kg and Idarubicin (1.5 mg/kg) plus Lentinox® at 20 mg/kg were administered orally to rats that were then later injected with BNML leukemia. Both studies had negative (saline) and positive (Idarubicin only) controls, as well. All rats were observed for weight loss, lethargy and hind limbs were monitored for paralyses. In addition, the Lentinox® groups were monitored for toxic effects, ataxia and behavioral changes. Survival of the BNML rats was the primary outcome variable. The rats that received the Lentinox® did not exhibit any adverse effects, and had survival times equal to the Idarubicin control groups. The combination of Lentinox® plus Idarubicin had slightly (not significant) lower survival times than the Idarubicin or Lentinox® only groups, by 1 day for the 0.5 mg/kg Lentinox® plus Idarubicin dose, and 1.5 days for the 20 mg/kg Lentinox plus the Idarubicin dose.

In a study conducted at Department of Veterinary Medicine, Louisiana State University (sponsored by MediMush), twenty-four mice were divided into four groups. Group 1 received 2 mg/kg Lentinox® in 0.15 M NaCl, intraperitoneally (i.p.), Group 2 received an equal volume of saline i.p., Group 3 received 10 mg/kg Lentinox® in water by gastric gavage (oral) and group 4 received an equal volume of water orally. All mice received treatment once daily for 5 days. Twenty four hours after the last treatment the mice were bled and ethanized for cell isolation. It was concluded that Lentinox® had no effect on the health of the mice and no statistically significant effect on their growth as measured by weight gain. However, i.p. Lentinox® had an effect on the number and phenotype of the cells isolated from the peritoneum lavage. Orally exposed mice and those that received saline i.p were relatively similar; however, there was an increase in the total number of cells isolated from the peritoneum of i.p. Lentinox® treated mice. The increased number of cells was due mainly to an increase in neutrophils in the population. The relative contribution to the

observed results by the PMN compared to macrophage cannot be estimated at this time, but might affect the difference observed differences in the i.p. groups. In contrast, the number and phenotype of the oral groups were very similar and their comparisons should be statistically valid. Intraperitoneal (i.p.) lentinan treatment caused a 4-fold increase in TNF α production at 12 hours by peritoneal exudate cells (PEC) and a 3-fold increase by splenocytes as well as increasing the amount of TNF α produced in response to LPS treatment in vitro by PEC (30% increase) and splenocytes (2-fold). Oral Lentinex $\text{\textcircled{R}}$ treatment had no significant effect on TNF α production by PEC or splenocytes. Interestingly, there was a modest but significant decrease (20%) in TNF α production by PEC following in vitro LPS stimulation.

These results indicate that the effect on TNF α may be initiated at the local level and/or require direct exposure to Lentinex $\text{\textcircled{R}}$. Both oral and i.p. Lentinex $\text{\textcircled{R}}$ treatment significantly enhance IL-1 α production by PEC (18-fold and 6-fold, respectively). In addition, IL-1 β in response to in vitro LPS was also increased more than 3-fold in i.p. treated mice. Like TNF, IL-1 β production by PEC from mice exposed to oral lentinan and stimulated in vitro LPS was decreased (2-fold). Splenocytes from treated and untreated mice produced small amounts of IL-1 β in the first 12 hours of culture. When mice were exposed to Lentinex $\text{\textcircled{R}}$, either orally or i.p., splenocytes from the mice were stimulated to produce IL-1 β by treatment with either LPS or ConA (a T-cell mitogen), however the production was very low compared to PEC. In general, lentinan treatment had only small effects on IL-6 production by PEC, although this was only significant in the non-LPS treated cells. In contrast, IL-6 production was modestly elevated by unstimulated splenocytes but was modestly decreased by LPS or Con A stimulated splenocytes, both oral and i.p. exposure. IL-6 levels continued to rise over the next 12 hours of culture. Interleukin 12 production at

12 hours was at the limit of detection of the assay except in ConA stimulated splenocyte cultures. In the i.p. lentinan exposure, mice produce a modest but significant amount of IL-12 in response to LPS. Oral Lentinex® exposure generally decreased pro-inflammatory cytokine production by PEC and increased pro-inflammatory production by splenocytes suggesting the systemic response to Lentinex® (oral) may be different from the local responses measured in previous studies (nasal, i.p., i.v.).

Two additional studies were conducted by MediMush at the Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences. In the first study, 360 male broiler chickens were fed different experimental diets containing combinations of 0.01, 0.1 mg or 1.0 mg of Lentinex®/kg of feed (in a preparation called LentiGuard) and/or a coccidiostatic agent (Monteban®). The objective was to study the effect of LentiGuard® in diets for broiler chickens. Weight gain did not differ significantly between any of the dietary treatments in any period. A numerical higher weight gain was observed for birds fed on diets with Monteban®, and birds on the treatment with the highest level of LentiGuard showed the second highest weight gain. Because of the similar feed consumption among the treatments, the feed/gain ratio did not differ significantly between the treatments. No clinical problems related to dietary treatments were observed. No significant effects of Lentinex® on performance, mortality and litter dirtiness were observed.

The second study involved the feeding of three different levels (0.01, 0.10, or 1.0 mg/kg feed) of Lentinex® (delivered as LentiGuard®) or a control diet for four weeks, to 32 weaned piglets per group. On average, the pigs in the three Lentinex® groups consumed 7.5 mg, 77.8 mg and 797 mg Lentinex® respectively. The objective was to study the effect of LentiGuard in diets for weaned piglets. No health problems related to the dietary treatments were observed. The piglets revealed

good growth relative to what is found at commercial pig farms in Norway, on average for all of the pigs the daily gain (ADG) was 559 g. The piglets generally showed good viability, which was evaluated subjectively. Especially in Period 1, the piglets fed the highest level of LentiGuard® (2.0 mg/kg) were found to have a good viability compared to the other groups. There were no significant differences among the dietary treatment groups in either weight gain or daily feed intake of piglets during week 1-2, week 3-4, or the overall piglet period. During the two first weeks, the pigs fed the 2.0 mg LentiGuard®/kg diet numerically gained 12% faster relative to the control diet. During the two last weeks, and the overall piglet period, the differences among the treatment groups were small. The addition of LentiGuard® to diets had a significant effect on several blood haematological parameters including the index of anisocytis, neutrophilic granulocytes and lymphocytes. A significant positive linear effect of increasing dietary levels of LentiGuard® was also found for haemoglobin and haematocrit.

Appendix 2

Safety studies related to Lentinan

The consensus of the scientific community is that Lentinan within defined dosage quantities is a safe compound for all species studied, is safe when taken chronically; and is safe regardless of the mode of administration. This consensus, especially with regard to oral administration, was achieved after Lentinan had been extensively studied during the 1980s (Wasser, 2005).

Safety studies, most of which have been published in peer-reviewed journals, included acute toxicity determination, subacute or subchronic toxicity determination, chronic toxicity determination, effect on fertility and reproduction and determination of teratogenesis. Lentinan was administered orally, by intravenous injection, by intraperitoneal injection, subcutaneous injection, intranasally and by inhalation. The effect of Lentinan administration was studied in several species, including mice, several strains of rats, guinea pigs, chickens, New Zealand white rabbits, beagle dogs and Rhesus monkeys. Most of these studies were rigorously controlled “pre-clinical” trials conducted by researchers from different countries in the Far East and in the West, in support of potential drug development programmes. (Full details of these safety studies can be included if considered relevant to the application).

This safety consensus eventually led to the commercialisation of Lentinan products throughout Asia and since the early 1990s in the United States where it is being sold as a dietary supplement under the auspices of DSHEA.

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