Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

Opinion on the safety of BioProtein®
by the Scientific Panel on Animal Feed
of the Norwegian Scientific Committee for Food Safety

Adopted on 20 March 2006

SUMMARY

BioProtein® (BP) is a trademark for single cell (bacteria) protein, based on conversion of methane, with the addition of ammonia and oxygen, to a protein source. BP is produced by Norferm AS in Norway, and has been authorised since 1995 for fattening pigs (8%), calves (8%) and salmon (19-33%). Significant immune effects were revealed in a toxicity study performed in rats fed a nucleic acid reduced BP product (NABP) and thereafter, similar effects were also found after feeding with untreated BP, although to a lesser extent. Additional studies confirmed increased mesenteric lymph node weights in cats and foxes. Due to the undesirable effects and also due to applications for extended use, BP has been assessed by the Scientific Committee on Animal Nutrition (SCAN) and EU's Scientific Committee on Food (SCF) in 1995, by SCAN in 2001 and 2003 and by the European Food Safety Authority (EFSA) in 2005. The EU memberstates United Kingdom, France and Finland have also made assessments. The approval from 1995 remains unchanged.

The Norwegian Food Safety Authority has requested the Norwegian Scientific Committee for Food Safety to assess the risk of using BP as a protein source in feedingstuffs, both for the animal categories already authorized and for an applied extended use to pet animals, chicken and pigs from weaning to slaughter. The Norwegian Scientific Committee for Food Safety is asked to consider all existing documentation.

BP is composed of a protein with a somewhat different amino acid compostion compared to fish meal, with relatively high level of nucleic acids, phospholipids, lipopolysaccarides and minerals. Effect studies with BP have been conducted in rats/mice, pigs, chicken, cats, foxes, and salmon. Most of the concern regarding the side effects of BP in feed is related to the immune response. The main findings included changes in weight and morphology of mesenteric lymph nodes, followed by induction of specific antibodies. Histopathological examination after feeding with NABP also revealed changes in the intestines and several internal organs indicating systemic effects. The rat trials with the ordinary BP product did not include an examination of the intestine. The Petitioner claims that the immune response seen in BP-fed mice/rats is most likely a normal response to ingestion of large doses of a foreign antigen, and further, that oral tolerance towards this protein is induced over time. However, these interpretations are not adequately supported by the supplied documentation. A tendency towards adaption might be indicated in some of the studies, other results argue against tolerance induction.

It is unclear whether the content of phospholipids, lipopolysaccarides, nucleic acids or the protein structure, or combination of these compounds are responsible for the immunological changes observed. However, the particulate structure of BP has been shown to influence the observed immune response as the systemic immune response was avoided by ingestion of BP free of whole cells.

The studies conducted in target species have not included adequate examinations of the immune effects from ingestion of BP. No histopathological effects were observed in the pig, chicken and salmon studies. Increased mesenteric lymph nodes were found in cats and foxes fed BP. In the remaining studies the main focus has been on production parameters; weight gain, feed intake, feed efficiency, observation of clinical health, and product quality. When the contents of amino acids were balanced, the inclusion of low levels of BP (up to 9%) tended to stimulate growth in pigs and the same tendency was found in chicken with up to 6% BP. Higher feed levels of BP increased the risk of the opposite effect. In salmon, a dose dependent improvement of growth was reported from a short term experiment (8 weeks). However, in a longer term experiments with salmon, depressed growth and increased liver weight were observed in freshwater at 19% BP and a tendency of reduced growth in saltwater at 20% BP. These results indicate effects at BP levels considerably lower than those currently approved (19 and 33%, respectively, in feed for salmon in fresh and saltwater, respectively). It can be argued that the inclusion levels of BP should be reduced for salmon.

Until possible effects in the immune system have been satisfactorily examined an inclusion level of 6% BP in the diet both to salmon and terrestrial target animals would reduce the risk of potentially adverse effects in the animals.

The risk associated with the human consumption of products from animals fed on BP is considered negligible. However, the production of single cell protein for feed production represents a relatively new scientific approach which implies precautionary handling.

KEY WORDS

BioProtein, Single Cell Protein, animal safety, food safety, health, immune effect

ABBREVIATIONS

BP	Bioprotein
NABP	Nucleic Acid reduced BP product
SCP	Single Cell Protein
EFSA	European Food Safety Authority
SCAN	Scientific Committee for Animal Nutrition (European Commission)
SCF	Scientific Committee for Food (European Commission)
AA	Amino Acid
MHC	Major Histocompatibility Complex molecules
GIT	Gastro-Intestinal Tract
TLR	Toll-like receptors
LPS	Lipopolysaccharides
ADG	Average Daily Gain
ADFI	Average Daily Feed Intake
F:G	Feed:Gain ratio

TABLE OF CONTENTS

GENERAL BACKGROUND AND EVALUATION BY OTHER BODIES

In the recent decades there has been an increased interest in the industrial production of microbiological protein for use in human and animal nutrition. The driving force has been the shortage of low cost protein sources in view of the increasing human population and living standards; resulting in famine in some parts of the world and increased demand for animal protein in others.

BioProtein® (BP) is a trademark for single cell (bacteria) protein, based on conversion of methane, with the addition of ammonia and oxygen, to a protein source. BP has been authorised since 1995 for fattening pigs weighing 25 to 60 kg, calves weighing above 80 kg and salmon. Statoil and DuPont have the last years been the owners of Norferm AS in Norway, the only plant producing BP, with an annual production capacity of 10,000 tons.

In 1998 the Petitioner asked for an extension of the authorisation of use of BP for fattening chickens and pigs to slaughter weight. In a supplementary dossier, significant immune effects were revealed in a toxicity study performed in rats fed a nucleic acid reduced BP product. These results triggered a re-evaluation of the previous studies of BP feeding. The Scientific Committee on Animal Nutrition (SCAN, 2001), verified that the immune effects seen after feeding with nucleic acid reduced BP also were present after feeding with untreated BP, although to a lesser extent. Additional studies confirmed increased mesenteric lymph node weights in cats and foxes, but not in pigs. Thus, these effects seemed to be species specific. It was concluded that the effect seen after feeding with BP was not satisfactorily explained with regard to animal safety.

Following the SCAN opinion 2001, the Petitioner submitted new documents and studies to clarify the safety concerns expressed by the SCAN, in particular, the effect on mesenteric lymph nodes. The SCAN adopted a new opinion in 2003 (SCAN, 2003), and concluded that the new information submitted by the Petitioner did not provide sufficient scientific evidence to alleviate the concerns expressed previously.

In 2005, the European Food Safety Authority (EFSA) adopted an opinion (EFSA, 2005) in light of the most recent SCAN opinion, taking into consideration new information provided by the Petitioner.

In light of the SCAN and EFSA scientific opinions on BP, no extended authorisation has been granted concerning other animal species.

Below is a more detailed description in chronological order of previous assessments and reports on the use of BP in animal feed from SCAN, EU's Scientific Committee on Food (SCF), EFSA, as well as from the EU memberstates United Kingdom, France and Finland. In addition, scientific evaluations of BP have also been conducted by the National Hospital, Oslo, Norway and the National Veterinary Institute of Norway.

SCAN and SCF, 1995

From the documentation provided, the SCAN and SCF concluded (SCAN/SCF, 1995) that BP was considered to have an acceptable, but not exceptional, value as a protein source in animal nutrition. A growth depression was observed in some target species after feeding with high concentrations of BP. A maximum of 8% of the diet was recommended for growing pigs from 25 kg to 60 kg and veal calves starting at 80 kg. For salmon in fresh and seawater the maximum levels were set at 19% and 33% respectively. The product was not considered to carry any microbiological or appreciable risk to livestock, provided that the maximum level of incorporation was not exceeded. No risk was identified to the consumers of the products of animals fed the protein source and there was no adverse effect of BP on the organoleptic

quality of the animal products. In common with most other protein products, it was considered a potential health risk for workers, due to sensitation by inhalation followed by possible development of respiratory allergic reactions in susceptible individuals.

SCAN, 1999

The background for this new evaluation was an application for the extension of use for piglets and fattening pigs to slaughter weight and chickens. A change in the production process led to a doubling of the growth rate with a subsequent increase in the concentration of nucleic acids, from approximately 7 to10%. For some of the target species (pigs and chickens) BP was considered to have a high biological value and a well-balanced amino acid profile. BP has relatively high contents of iron and copper, and consequently the copper content gave rise to a recommendation that the product should not be fed to lambs or sheep.

The SCAN concluded (SCAN, 1999) that the product was well tolerated and utilized by pigs and poultry. The inclusion of 8% BP in the diet for pigs from weaning (28 days) to slaughter would not affect health or growth rate. However, higher inclusion levels tested could result in depression of growth and feed conversion efficiency. The recommended inclusion level for chickens for fattening was 6%.

SCAN, 2001

This evaluation (SCAN, 2001) was initiated as new toxicological data on a nucleic acid reduced BP (NABP) revealed immune effects in rats. In addition, the Petitioner applied for extended use of BP to include pet animals.

The background for the development of a nucleic acid reduced BP product was a putative extended use of BP in human diets. The high content of nucleic acids (approximately 10%) in BP is not considered to be a problem in animal nutrition, but humans are more sensitive to high concentrations of nucleic acid.

The SCAN concluded that the immunological reactions observed in the rat studies with NABP did not have a profile consistent with a development of a “normal” immune response or oral tolerance. The SCAN considered the inflammatory responses seen after feeding with BP adverse and unacceptable, and did not recommend an extension of use of the product.

SCAN 2003

New reports were provided by the Petitioner to demonstrate that the immune responses to BP are inadverse, and just a normal response to a foreign antigen; i) antibody production against BP was observed in the parent rats but not in their offspring, ii) substituting BP with Brewers Yeast showed a similar production of antibodies against Brewers Yeast, which increased with prolonged exposure. The Petitioner concluded that antibody production increased with age, and was a result of a normal immune response against the ingestion of large quantities of foreign proteins. The Petitioner claimed that their results indicated an induction of oral tolerance towards the proteins.

The SCAN raised questions regarding the specificity of the detected antibodies. The increase in antibody production with age indicated a persistent stimulation and raised questions as to the suitability of using BP in feed. The included Brewers Yeast feeding study in rats demonstrating similar histopathological changes as observed after feeding with BP: enlarged mesenteric lymph nodes and spleen, and Brewers Yeast specific antibodies was interpreted as the changes observed after feeding with NABP and BP were not fortuitous, but appeared to be associated to single cell proteins. The SCAN concluded that the applicant had provided inadequate scientific evidence as to describe the nature of the observed effects on health

parameters, and insufficient information to demonstrate the safety of BP as a protein source in feeding stuff.

EFSA, 2005

The most recently submitted data included a single generation reproduction study using BP and Brewers Yeast (Takawale, 2004a), a study with juvenile rats fed BP (Takawale, 2004b) and antibody studies in parent and young rats fed BP, with or without immunisation with BP (Thestrup 2004b).

No adverse effects of BP were observed on reproduction performance, growth and development in the groups fed BP or Brewers Yeast. The effects on mesenteric lymph nodes were less prominent in offspring rats fed BP compared with the parents. The antibody studies showed lower antibody responses to BP in the offspring fed BP compared with parent rats fed BP and lower antibody responses in BP fed rats compared to casein fed control rats after intraperitoneal immunisation with BP in Freund's incomplete adjuvant. The EFSA Scientific Panel (FEEDAP) concluded (EFSA, 2005) that the results confirmed the effects described in the earlier studies of the effect of BP on the immune system in rats. The Scientific Panel did not consider that the results in old rats, antibody production and non-specific immunity (effects on macrophages and neutrophils), could be interpreted as indicative of tolerance. The inclusion of Brewers Yeast in the feeding trials of rats did not, in their opinion, provide any further insight into the explanation of the adverse effects. The Panel concluded that these studies indicated that in animals fed BP, the effects on haematology, lymphoid organs and antibody production under specific circumstances (feeding at very early age or after immunisation) were diminished or partially reversible. These observations indicated that the effects are at least partially subjected to adaptation, however, they do not alleviate the concerns for animal and human safety.

Central comments and questions raised by EU-countries

1) France, 2005 (Direction generale de la concurrence, de la consummation et de la repression de frauds, Ministère de L'economie de finances et L'industrie (France, 2005a), and Agence Francaise de securite sanitaire des aliments (AFSSA) (France, 2005b).

Concerning cats: In their opinion the studies did not cover the whole life span, only a short term uptake study has been performed in 10 cats. The protocols and presentation of results were considered insufficient and no urine quality studies were performed (risk for urine lithiasis, due to high nucleic acid content).

Concerning dogs: Only results from feeding trials with farmed blue foxes have been presented and there is an obligation to present results from trials performed on the target species.

Concerning pigs: The studies were considered of too short duration and comprised few animals.

Concerning salmon: The report was considered incomplete and the results preliminary.

2) United Kingdom, 2005 (Food Standards Agency, FSA)

The FSA concluded (UK, 2005) that the effects observed in rats on lymph nodes, liver, spleen and haematological changes appear to have an immunological aetiology. The same adverse effects may also affect target species. New documentation, a study using soya rather than BP and, a pig tolerance study – looking superficially at health effect, did not, in their opinion, demonstrate absence of immunological effects in the target species. The FSA considered that further work was needed to demonstrate that the effects seen in rats are not adverse – for example to characterize the changes occurring in the immune system. The FSA did not

approve of the Petitioner's approach trying to show that common dietary proteins have similar effects and requested that demonstration of safety for consumers of meat from BP fed animals.

3) Finland, 2005 (Agriculture Chemistry Department, Plant production inspection centre)

The Finnish evaluation (Finland, 2005) concluded that BP seems to act as an immunostimulant. No harmful effect on health or reproduction capacity in cats and rats were observed, but there was no documentation on the safety for dogs.

Central comments and questions raised by Norwegian human and veterinary medicine institutions

The National Hospital, Oslo Norway. Commissioned by Norferm.

The report (Brandtzaeg *et al*., 2001) describes a histopathological evaluation of mesenteric lymph nodes from several species (pig, fox, cat, rat) fed BP. However, it is not indicated which studies the evaluation is based on.

Dramatic size increase is frequently observed in human mesenteric lymph nodes as immunological response in the absence of overt disease. Thus, the moderate increase in weight of mesenteric lymph nodes observed in the BP feeding experiments would be considered as rather negligible in a clinical diagnostic setting. Mesenteric lymph nodes are unique because their afferent lymph drains both GALT structures, such as Peyer's Patches and intestinal lamina propria. It was not considered possible on the basis of a histological evaluation alone to conclude whether the reactive changes observed after feeding with BP reflect an induction of active (protective) immunity, tolerance or both. The minimal pathological changes observed after feeding were considered of no concern. They found no histological evidence that the mesenteric lymph nodes of animals were adversely affected by feeding with BP in the amounts and over the time periods of the experiments – and considered the reactive changes observed as a normal mesenteric lymph node response to foreign antigens from the gut lumen. Thus, these data suggest that BP does not behave differently in this respect compared to other exogenous low-grade or dietary microbial stimuli.

National Veterinary Institute, Oslo, Norway. Commissioned by The National Agriculture Inspection Service, Norway.

Two documents from the National Veterinary Institute of Norway (dated 16.11.2000 and 17.12.2003) evaluate BP and NABP (Fossum & Næss, 2000, Fossum & Gudding, 2003).

The evaluations concluded that NABP most likely induces an immune response without any documented development of tolerance. The changes observed in the lymph nodes indicate that the immune response provoked a hypersensitivity reaction. The significance of the changes observed is uncertain, but is of particular importance in young animals where the immune system is not fully developed. Risks associated to human consumption of meat from animals fed BP was not identified.

TERMS OF REFERENCE

Several scientific opinions exist to date on the safety of the use of of BioProtein as protein source for various animal categories. In addition there are responses from the Petitioner to several of these opinions. The Norwegian Food Safety Authority has requested the Norwegian Scientific Committee for Food Safety to assess the risk of using BP in feedingstuffs, both for the animal categories already authorized and for the applied extension of use. The Norwegian Scientific Committee for Food Safety is asked to consider all existing documentation.

ASSESSMENT

The Panel of Animal Feed in the Norwegian Scientific Committee for Food Safety established an ad hoc-group to work out an opinion with the following mandate:

- Consider the results of the studies performed
- Consider the quality of the studies
- Do a risk assessment regarding animal health
- Do a risk assessment regarding human health when exposed through animal products
- Point out lack of knowledge or need for new studies if necessary

1 Introduction

1.1 Production of BioProtein

Microorganisms, for example yeast and lactic acid bacteria, have been used in human nutrition for thousands of years. In the last decades there has been an increased interest in the industrial production of microbiological proteins for human and animal use. The driving force has been a possible shortage of low cost protein sources.

Technology developed during the last century has made large-scale production of cells from fungi and bacteria possible. The products from such fermentation are commonly called single cell protein (SCP), but contain in addition to proteins also nucleic acids, lipids, carbohydrates, vitamins and minerals. The minor constituents vary according to the type of microorganism, substrate on which the organisms is cultivated and the processing conditions.

Several different microorganisms have been used to produce SCP (Punia and Singh, 1995; Anupama and Ravindra, 2000). However, only a limited number of organisms have been used in commercial products including: Mycoprotein (*Fusarium*), Toprina (*Candida*), Pekilo (*Paecilomyces variotii)*, Pruteen (*Methylophilus methylotrophus*), Quorn (*Fusarium*) and BioProtein (*Methylococcus capsulatus*). Only two of these products are produced with prokaryotic organisms: Pruteen and BP.

The production of BP directly from methane is a biotechnological process converting methane in to a protein source for feeding animals, including fish. The methane oxidising organism, *Methylococcus capsulatus*, is cultivated under hygienic conditions in a steel fermentor. In addition to methane, oxygen, ammonia and minerals are added. Due to impurities in the methane gas by longer alkanes (C_2 and C_3) and lysis of some of the bacterial cells, other types of prokaryotic organisms are added to the fermentor to remove these impurities. *Alcaligenes acidovorans* (*Delftia acidovorans*), *Bacillus firmus* and *Bacillus brevis* are the bacterial cultures added to the fermentation process. The strain *B. brevis* NCIMB 13288 used in the process was reclassified to *Aneurinibacillus danicus* in 2004. In the final fermentation product 88 % of the cells originate from *M. capsulatus*, 11 % from *A. acidivorans* and 1 % from *B. firmus* and *A. danicus*."

The main constituent in Bipoprotein is the bacterium *Methyllococcus capsulatus* Bath. This bacterium belongs to a group of obligate aerobic, methane oxidizing bacteria. These bacteria can be isolated from all parts in nature where methane and oxygen coexist. Isolations have been made from, marine-, fresh water- and terrestrial habitats. These habitats vary form acidic to alkaline and in temperature up to 55 °C. *Methyllococcus* is a group of bacteria widely distributed in nature (Lidstrøm, 1992).

Methylococcus is thermo tolerant or moderate thermopile and can be cultivated at temperatures ranging from 40 – 60 °C.

M. capsulatus have usually spherical cells. The cell wall is typical for Gram-negative bacteria. The bacterium can grow on substrates with only one carbon atom. Both nitrate- and ammonium salts (including ammonia) work well as nitrogen source. The bacterium does not require growth factors. The cells contain an elaborate intracytoplasmic membrane structure which is important for the assimilation of methane. This membrane system and necessary enzymes for oxidation of methane, seems to be dependent upon available cupper in the growth medium (Bowman, 2005).

The recent completion of the genome sequence of *M. capsulatus* Bath is an important event in molecular microbiology. This tool will make it possible to modify key enzymes in the methane fixating bacterium making it a more commercially adapted bacterium in the production of single-cell protein (Kelly *et al*. 2005).

A. acidovorans is a gram-negative bacterium that has soil and sediments as its natural habitat. This bacterium has from time to time been isolated from the gastrointestinal tract of mammals as a result of the individual´s contact with the environment and the resultant establishment of the bacterium as part of the normal flora of the animal for varying time periods.

B. firmus is a gram-positive bacterium that is isolated from agricultural soil under different management regimes and therefore is a regular organism in the vicinity of both production animals and pets.

A. danicus is a gram-positive bacterium that is related to soil. Until recently it belonged to the heterogeneous *Bacillus brevis* group with members isolated from soil and of which several have been utilized in bioprocesses.

The bacterial broth product from the fermentation process are dewatered by centrifugation, ultrafiltration and drying. Under full capacity the production potential is 10,000 tons of BP per year.

1.2 Composition of BioProtein

1.2.1 Amino acids

BP is intended as a dietary protein source for salmon and terrestrial domestic animals. The amino acid composition of a protein source is of great importance and a comparison of the amino acid composition of BP, fish meal and soybean meal is presented in Table 1. The nitrogen content of BP is similar to fish meal, but considerably higher than in soybean meal. However, the protein content is significantly lower in BP than in fish meal and soybean meal, due to the high concentration of nitrogen containing nucleic acids in BP. The relative amino acid concentrations are shown in Table 1. BP and fish meal have more similarities in amino acid content than soybean meal. When the essential amino acids are summed up, BP and fish meal deviate 1-2%, and BP has 16% (11% for fish feed) higher levels of essential amino acids than soybean meal.

With regards to individual amino acids, the relatively low content of lysine in BP compared to fish meal (-22%) is the major concern since monogastric animals require an additional lysine supply. On the other hand, BP has a higher content of tryptophan in particular, than fish meal (69%). Furthermore, the isoleucine content in BP is more than 20% lower than in fish meal and soyprotein. BP typically contains 10% less methionine than fish meal but approximately almost 110% more methionine than soy protein. It is essential that these amino acids are balanced in animal diets as this can affect both health and carcass composition of the animals (Nissen 1992; NRC, 1994; NRC, 1998).

Feeding low levels of lysine to broilers and pigs are known to affect carcass composition since these animals store more lipid in muscle tissue (Witte *et al*., 2000; Vieira *et al*., 2004; Wijtten *et al*., 2004; Carew *et al*., 2005) compared to animals fed adequate amounts of lysine. Similar effects on carcass composition have been observed in Atlantic salmon fed on low dietary lysine (Espe *et al*., unpublished results).

Methionine is the main methyl donor within all cells (Finkelstein 1990; Mato *et al* 1997). When the dietary methionine levels varies this may affect the capacity of methylation and thus results in hyper- or hypomethylation in some tissues (Shoveller *et al*. 2004; Selhub, 1999), which may affect animal health. It has been shown that unbalanced methionine diets fed to rats affect adiposity and cholesterol and thus their health (Baba *et al*., 1992; Kern *et al*., 2002; Wergedahl *et al*., 2004).

Table 1. Nitrogen (g^{-1} kg dry matter) and amino acid (AA) total content as percent of N*6.25 and individual amino acids expressed as (g/100g analysed AA) in BioProtein (BP), fish meal and soybean meal. The percent deviations between BP and the respective other two protein sources commonly used in animal diets are also given.

	BP	BP	Fish meal[1]	BP	Soybean meal[1]
		% deviation from Fish meal	g/100g AA	% deviation from Soybean meal	g/100g AA
Nitrogen	120.96	-2	122.88	+50	80.64
Sum AA per N *6,25	84.8		98.6		97.8
Alanine	8.0	+28	6.2	+87	4.2
Arginine*	7.5	+13	6.6	0	7.4
Aspartic acid	9.8	-3	9.9	-14	11.1
Glutamic acid	12.1	-14	14.0	-35	18.2
Glycine	6.6	2	6.4	+54	4.2
Histidine*	2.4	-7	2.5	-15	2.7
Isoleucine*	3.8	-22	4.8	-20	4.6
Leucine*	8.8	+9	8.0	+15	7.5
Lysine*	6.4	-22	8.1	+2	6.1
Methionine*	2.8	-10	3.1	+113	1.3
Phenylalanin*	4.8	+16	4.1	-5	5.0
Proline	3.9	-13	4.4	-24	5.0
Serine	3.8	-21	4.7	-29	5.2
Threonin*	5.7	+21	4.6	+42	3.9
Tyrosine	3.9	+10	3.5	-0	3.8
Valine*	6.8	+20	5.6	+42	4.7
Cysteic acid	0.9	-7	1.0	-38	1.5
Tryptophan*	1.9	+69	1.1	+32	1.4
Essential AA	39.2 (49.1)	+1(2)	38,8 (48.1)	+16 (+11)	33.8 (44.2)
Non-essentialAA	60.8 (50.9)	-1 (-2)	61,2 (51,9)	-8 (-9)	66.2 (55.8)
EAA/NEAA	0.64 (0.96)	+1 (+4)	0.64 (0.93)	+26 (+22)	0.51 (0.79)
Sum AA	100		100		100

[1]From the Norwegian feed table: http://www.umb.no/iha/fortabell/ (Anon, 2006)

* Essential AA. In fish both arginine and histidine are also considered essential, thus fish have a requirement for 10 AA, while other species require 8 AA. Values in parentheses are for fish.

In conclusion, differences in amino acids ratios in diets may have important impact on health as well as the carcass composition of the farmed animals if not adequately balanced.

1.2.2 Protein

The mucosal lymphoid system in the gastro-intestinal tract is constantly exposed to a variety of foreign antigens (peptides) from foods, from commencal bacteria of the gut, and from pathogenic microorganisms and parasites, and must therefore decipher a large number of signals at all times. Responding correct to each set of signals is crucial.

No immune response can normally be detected to food antigens. Indeed, soluble antigens taken by mouth usually induce a state of unresponsiveness (mucosal tolerance). In contrast, when pathogenic microorganisms invade the intestinal mucosa, it is necessary to elicit strong T and B cell responses. The mucosal lymphoid system is therefore in the position of constantly fighting intolerance to food and the commensal flora, while effectively battling infectious microbes. Determining precicely which type of response to generate in each case, is the key to the prevention of immune dysregulation and tissue damage. The key distinction between tolerance and the development of powerful protective adaptive immune responses is the context in which peptide antigen is presented to T lymphocytes in the mucosal immune system.

In the absence of inflammation, presentation of peptides to T cells by major histo-compatibility complex (MHC) molecules on antigen-presenting cells occurs in the absence of co-stimulation. By contrast, pathogenic microorganisms induce inflammatory responses in the tissues, which stimulate the maturation and expression of co-stimulatory molecules on antigen-presenting cells. This form of antigen presentation to T cells favours the development of a protective T_H1 response.

It is demonstrated that a substantial number of proteins in the outer membrane (outer membrane sub-proteome) of *M. capsulatus* have relatively few parallels in other organisms studied (Berven *et al.* 2006). Purified antigens are, however, not usually strongly immunogenic on their own.

1.2.3 Nucleic acids

Single Cell Protein, such as BP, contains nucleic acid (DNA and RNA) at levels 10 to 100-fold higher than traditional foods and feeds. While many traditional foods contain in the order of 0.1 to 1% DNA ww, single cell protein contains 5 to 15% nucleic acids (eg. Quorn (from *Fusarium*) and BP (from *Methylococcus capsulatus*).

A major fraction of dietary DNA is degraded in the gastrointestinal tract and nucleosides are passively and actively absorbed (Kichenin and Seman, 2000). Most of the dietary DNA is catabolised and used as a source of energy. Reuse of nucleotides derived from dietary DNA into newly synthetised nucleic acids does also occur (Sánchez-Pozo and Gil, 2002). However, insufficient catabolism of nucleosides, or too high exposure in the diet may lead to precipitation of insoluble particles of uric acid (goat disease) in humans. Thus, the Protein Advisory Group (PAG) of the United Nations has set a daily exposure of 2 grams as the maximum safe human exposure level for the adult populations (PAG, 1975). Animals, however, catabolise nucleosides more efficiently into water-soluble metabolites, and consequently tolerate higher levels of nucleic acids. Dogs and cats may create urate uroliths following increased level of uric acid in the urine (Nelson and Couto, 1998). Some dog strains are particularly susceptible for this disorder due to decreased catabolism of uric acid.

Recently, intact dietary DNA, several hundred base pairs long, was found to be taken up from the gastrointestinal tract and distributed to blood and organs. Up to 0.1% of DNA fed to mice could be retrieved in the animals' blood (Doerfler, 2001) and fish blood (Nielsen *et al*., 2005; 2006). However, dietary DNA in blood undergoes continuous elimination and degradation. Based on available data, it has been estimated that approximately 1% of dietary DNA is

absorbed from the GIT (Nielsen *et al*., 2006). Thus dietary DNA, as intact DNA molecules, may have other effects than that of absorption of nucleotides.

Dietary nucleotides have also been shown to have a positive effect on immunity in mice (Carver, 1994). Further also dietary nucleotides influence lymphocyte maturation, activation and poliferation, especially in infants and enhance production of interleukin in intestinal explants (reviewed by Gil, 2002). Animal DNA has a different structure than bacterial DNA, which is high in unmethylated CpG sequences, and has been shown to elicit immunological responses, including activation of macrophages and dendritic cells. Moreover, such DNA also works as an efficient adjuvant (Oxenius *et al.*, 1999). It is thought that adjuvants act on antigen-presenting cells, especially dendritic cells. The effects of many adjuvants are mediated by activation of Toll-like receptors (TLR) on dendritic cells. Bacterial CpG DNA binds to TLR-9. The physiological role of TLRs is to stimulate an inflammatory and immune response to infection. When dendritic cells are activated through ligation of TLRs, they respond by secreting cytokines and expressing co-stimulatory molecules, which in turn stimulate the activation and differentiation of antigen-specific T cells. Dietary DNA has been shown to promote a shift in Th1 / Th2 balance towards Th1-dominant immunity in mice (Sudo *et al*., 2000). Free DNA from normal bacterial flora and infections (external sources) and cell degradation (internal sources), may also affect the immunological response to dietary antigens. A moderate dietary supplementation of nucleotides have improved disease resistance and enhanced the efficacy of vaccination in salmon (Burrells *et al*., 2001a; 2001b).

Only a limited number of experimental studies are available to resolve questions related to direct exposure to dietary DNA. Knowledge gaps identified by the ad-hoc group on Antibiotic Resistance Marker Genes of the Norwegian Scientific Committee of Food Safety include: lack of a detailed understanding of uptake mechanisms, transport pathways and degradation mechanisms and dynamics of food-derived DNA in the intestinal cells and bloodstream of mammals; lack of quantitative data on DNA exposure rates and fragment size distribution in the digestive system of various mammals digesting various foods and a lack of sensitive methodology and suitable model systems to address adequately the fate, and possible biological effects of food-derived genes entering the bloodstream of mammals. The Scientific Committee concludes that research into the basic understanding of the pathways, distribution and degradation dynamics of dietary DNA is necessary in order to strengthen the biological risk assessment of potential unintended effects caused by novel foods (VKM, 2005).

1.2.4 Phosholipids

Phospholipids are well known emulsifiers and are present in the protein particles formed during dewatering of the single cell proteins. BP consists mainly of *Methylococcus capsulatus*. The bacterium contains an elaborate membrane system which explains their ability to grow on methane. The concentration of crude fat for BP is given by Norferm as 8.5±1.1% on a dry weight basis, which is not significantly different from the lipid content in Gram-negative bacteria (7.5±3.1%). Membrane systems in organisms are mainly composed of phospholipids, and constitute approx 80-90% of the lipid content in bacteria not accumulating in depot fat (triacylglycerol). The type of phospholipids from *M. capsulatus* exhibits a typical gram-negative distribution with phosphatidylethanolamin (PE) as the major phospholipid species (Makula, 1978). The content of PE is 89% and the remaining phosholipid fraction is phosphatidylglycerol (PG), phosphatidylcholine (PC) and cardiolipin. The fatty acid composition is limited to 14:0, 16:0 and 16:1 (Fang *et al.* 2000), and these fatty acids are usually found in biological material and are not considered to constitute any concern regarding safety. In comparison, fish meal contains 2-4% phospholipids (personal

communication Jan Pettersen, Fiskeriforskning) and soybean meal less than 0.5% (personal communication Tor Kristoffersen, Denofa).

In mink fed a diet with natural gas utilizing bacteria the plasma level of PE was similar as in mink fed a soyebean diet, but the plasma concentration of homocysteine was significantly higher in the bacteria fed mink (Muller *et al*. 2005). The authors describe an enzymatic phospholipid methylation reaction generating homocysteine, and indicate that increased levels of homocysteine may be involved in mechanisms leading to vascular diseases.

1.2.5 Lipopolysaccarides

Endotoxins are an integral part of the cell walls of gram-negative bacteria and are liberated upon lysis. They are lipopolysaccharides (LPS), and the Lipid A portion is responsible for the toxicity. They often produce fever in the host and are fatal for laboratory animals at slightly higher doses than exotoxins (Powar and Daginawala, 1995). Single cell proteins (SCP) from *Pseudomonas* species and *Methylomonas methanica*, used in animal feed have been shown to cause febrile reactions and high titres of IgG and IgM due to the presence of endotoxins (Ekenvall *et al*., 1983).

Research results are linking lipopolysaccharides in gram-negative bacteria to a strong stimulation of the immune system of the gastrointestinal tract (GIT) (Calandra, 2003). LPS appear to play an important role in the development of postnatal immune activity of the GIT. Results from studies on germ-free animals and on pathological disorders of the GIT such as inflammatory bowel disease in humans indicate that LPS are important in maturation and in the pathological processes related to stimulation of the immune system in the GIT (Caradonna *et al.* 2000). Supplementing lactobacilli without LPS to individuals with inflammatory bowel disease appears to relieve the symptoms caused by the inflammatory factors of the immune system of the GIT (Caradonna *et al.* 2000).

LPS from gram-negative bacteria of the normal flora as well as from pathogens can be specifically recognized by the mammal immune system. This is due to the presence of external sugar residues on these LPS. The normal flora of neonates is important in the maturation of the immune system i. e. the development of tolerance to the microorganisms in the normal bacterial flora (Caradonna *et al.* 2000).

As mentioned above, most adjuvants act on antigen-presenting cells as dendritic cells through pattern recognition receptors such as Toll-like receptors (TLR). LPS has adjuvant effects, but these are limited by its toxicity. LPS and lipid A are ligands for TLR-4, which seems to be the most important receptor mediating the adjuvant effect of LPS and its derivatives.

Certain polysaccarides as β-glucans are utilized as immue stimulants in aquaculture and terrestrial farm animals (Robertson *et al*., 1994; Shoenherr *et al*., 1994).

1.2.6 Minerals

The SCAN raised concern regarding the iron concentration in BP (SCAN, 1999). The iron concentration in BP is 325 mg/Kg (Thestrup, 2004a). This is the similar level of iron as in other protein sources for feed-preparations as soybean meal and in fish meal with iron concentrations of 315mg/Kg and 305mg/kg respectively (CVB, 2003). However, a much higher content of iron was measured in NABP (nucleic acid-reduced BP), 3200 mg/Kg (Thestrup, 2004a).

The concentration of copper in BP is 105 mg/Kg (Thestrup, 2004a). In comparison, fish meal and soybean meal (extracted) contains copper levels of 6 and 15 mg/Kg respectively (CVB, 2003). The relatively high copper content in BP makes it unsuitable for use as a feedingstuff for animals that are sensitive to copper such as sheep (SCAN, 1999).

1.3 Occurrence of process developed undesirable substances?

The production process for dry single cell protein (SCP) includes centrifugation, ultrafiltration and spray drying of dewatered cell-mass. None of these processes expose the BP to high temperatures for considerable time which could have implied a risk for temperature developed contaminants.

The high content of iron in NABP (nucleic acid-reduced BP) indicates an addition of iron, intentionally or unintentionally, during this production process.

1.4 Statutory limits and use

The EU approval of BP is founded in Council Directive of 30 June 1982 concerning certain products used in animal nutrition (82/471/EEC) with amendment in Commission Directive 95/33/EC of 10 July 1995. The animal species are pigs for fattening from 25 to 60 kg, calves from 80 kg on, and salmon, and the maximal incorporation rates in the feed are 8% for pigs and calves, 19% for salmon in fresh water and 33% for salmon in seawater.

In total, about 10,000 tons have been used as salmon feed, while BP is not commercially used for pigs and calves. A large-scale plant, capable of producing 10,000 tons per year came on stream in 2001. Reduced sale and lack of extended EU approval have resulted in temporary stop of BP production and at present, there is no use of BP as a feed ingredient.

2 Critical evaluation of the present data

2.1 Studies with laboratory animals

Several studies, some supplied by the petitioner, others published in peer-reviewed journals, have been performed to try to elucidate the pathological and immunological effects of feeding laboratory animals with a diet containing BP. Both untreated BP and nucleic acid reduced BP (NABP) have been tested. The studies considered relevant for evaluating the effects of feeding with BP on the immune system in laboratory animals are assessed below.

2.1.1 Immunotoxicity of nucleic acid reduced BioProtein – a bacterial derived single cell protein – in Wistar rats (Mølck *et al.,* 2002)

The study is published in *Toxicology*.

2.1.1.1 Study design

A subchronic toxicity study was designed to establish the safety of feeding with NABP (a product developed for putative human consumption). Four groups of Wistar rats (each of 10 female and 10 male, aged 6-7 weeks) were fed with diets including 0, 6, 12 and 24% NABP over a period of 90 days. Blood and urine samples were collected both before feeding with NABP and in the last week of the study. At the end of the trial, all animals were sacrified for gross and histopathological examination.

2.1.1.2 Observed effects

No effects were observed on general health or mortality in rats fed diets containing NABP. Histopathological changes were observed in the following organs from NABP-treated animals: mesenteric lymph nodes, intestine, spleen, liver, bone marrow in sternum and female

kidneys. The most obvious changes were observed in the mesenteric lymph nodes, where dose-dependent increases were observed in their relative and absolute weights, histiocytosis and accumulation of foamy macrophages. The main changes in the intestine comprised increased infiltration of eosinophilic granulocytes in lamina propria of the small and large intestine, and intracellular iron containing pigments in the colon. In the liver, hyperplasia of Kupffer cells was observed. Moreover, feeding with NABP induced specific humoral responses (IgM and IgG) against the protein.

The authors conclude that NABP seems to have greater impact on the immune system than other single cell proteins, and that further characterization of the type of immune response is needed, in addition to investigation of the effect of product processing (as the nucleic acid reduction process).

2.1.1.3 Evaluation and main conclusion for the risk assessment

This study presents a detailed histopathological examination of several tissues and demonstrates several dose-dependent specific immune related responses after feeding with NABP, while no effects on general health and mortality were observed. The trial did not determine which of the actual components in the NABP (lipids, proteins, carbohydrates, DNA/RNA or mineral content) induced the observed effects. The measurement of immune responses was limited to the detection of specific IgM and IgG antibodies and is preliminary and requires further studies.

2.1.2 The oral immunogenicity of BioProtein, a bacterial single-cell protein, is affected by its particulate nature (Christensen *et al., 2003*)

The study is published in *British Journal of Nutrition.*

2.1.2.1 Study design

The aims of these experiments were to characterize the type of immune response to feeding with BP and nucleic acid-reduced BP (NABP), its development over time and product-related causative factors. In one feeding study over 8 weeks, five groups of five mice (female BALB/c A, 9-11 weeks old) were fed with NABP (6 and 24%), untreated BP (24%) or casein (24%; control). The last group were fed NABP (24%) for 2 weeks and then fed a control diet. In a separate study, three groups (six mice in each group) were fed for 4 weeks 24% untreated BP or a control diet, or a control diet with cell-free BP-culture homogenate added to the drinking water (corresponding to a protein intake from BP equal to 24% in diet). Blood and saliva samples were collected weekly, and BP-specific antibodies (IgM, IgG1, IgG2a, IgA) were determined by ELISA. An *in vitro* cell proliferative assay was performed using mice spleen cell suspensions and ^{3}H-thymidine incorporation, and BP-culture homogenate was run on SDS-PAGE followed by immunoblotting.

2.1.2.2 Observed effects

Ingested BP induced a persistent mucosal and systemic immune response and a dose-dependent production of IgG and IgA in blood (systemic response) and IgA in mucosa (local response). The immune responses to NABP feeding were similar to the responses to untreated BP. The immune response was T-cell dependent, as specific splenocyte proliferation took place upon *in vitro* stimulation with BP. The humoral response was characterised by an initial rapid IgM production followed by a much greater increase in IgG level. The IgG2a response was selectively sustained in contrast to the abating IgG1 response subsequent to termination of feeding on the BP diet, indicating a Th1 cell response. The induction of a specific systemic response to BP was avoided by administration of BP in a whole-cell free preparation.

2.1.2.3 Evaluation and main conclusion for the risk assessment

Here, a more detailed and highly relevant characterisation of the immune response towards ingested NABP and untreated BP is described. Feeding with BP induces a specific Th1 response, as the IgG2a response was selectively sustained in contrast to the abating IgG1 response subsequent to the termination of feeding on a BP containing diet. The results suggest that there is no induction of tolerance, where a Th2 response bias is considered favourable. Ingestion of BP was found to induce a systemic humoral response, but this was avoided by feeding with a whole-cell-free BP preparation. This indicates that the physical preparation of single-cell proteins, such as BP, intended for animal consumption is indeed an important factor beyond the components per se – for the immune response induced upon ingestion.

2.1.3 BioProtein – One-generation reproduction toxicity study in the rat (Clausing & Bøgh, 2002).

The study was sponsored by Norferm and reported in a test report

2.1.3.1 Study design

The study was designed to determine the effects of BP on male and female reproductive performance including gonadal function, oestrous cycle, mating behaviour, conception, pregnancy, parturition, lactation, weaning and the growth and development of the offspring up to weaning. Ninety-six male and 96 female Wistar rats aged 5-6 weeks (males) or 8-9 weeks (females) at the start of the treatment were placed in four groups of 24 animals of each sex. The animals were treated orally by dietary administration of casein (Group 1, control), 5.5% BP (Group 2), 11% BP (Group3), or 22% BP (Group 4). Males were treated 10 weeks before mating and during the mating period. Females were treated from 2 weeks before mating and until weaning the offspring on postnatal day 21. Selected male offspring from group 1 and 4 were given either the control diet or 16.5% BP diet for until 12 weeks from weaning. At necropsy blood samples from all parental animals and selected offspring were collected for possible serological analyses (results not reported). Various reproductive tissues and the mesenteric lymph nodes were macro- and microscopically examined.

2.1.3.2 Observed effects

There were no effects of treatments on in-life reproductive or other parameters recorded, except that the feed consumption was dose-dependently reduced in both sexes of the parental rats, particularly during the first weeks of exposure.

The weight of mesenteric lymph nodes was significantly higher in parental females in the medium and high dose groups and a similar trend was observed in parental males. Microscopically, focal and multifocal granulomas were found in some of these lymph nodes. No such effects were found in the offspring at weaning or after until 12 weeks of feeding BP at a dietary concentration of 16.5% from weaning.

2.1.3.3 Evaluation and main conclusion for the risk assessment

The study was performed with three dose groups and a sufficient number of animals to demonstrate effects of BP in the diet on reproductive performance. Increased weight of and histological changes in the lymph nodes were demonstrated in parental animals, but not in offspring, which could be indicative of a development towards adaption. This study is however of limited value for determining what kind of immune response induced after feeding, as no such conclusion can be based on histopathological data alone. No serological results on immunoglobulin measurement were available. No histological examination of the instestine was performed.

2.1.4 Brewer´s Yeast - a combined 4- and 8 week lymph node toxicity study in the rat (Glerup, 2002)

The study was sponsored by Norferm and reported in a test report.

2.1.4.1 Study design

The study was designed to assess the possible effects of Brewers Yeast on the mesenteric lymph nodes, the iliac lymph node, spleen, liver, lungs and the gastrointestinal tract after daily administration in the diet. Sixty female SPF Wistar rats were allocated into three groups given a diet containing casein (control), or 11% or 22% of Brewers Yeast. Half of the animals in each group were killed after four weeks, the other half after eight weeks. The organ weight of the left iliac lymph node, the mesenteric lymph nodes, the spleen and the liver were recorded and the respective samples from these organs, the lungs and the gastrointestinal tract were subjected to histopathological analysis.

2.1.4.2 Observed effects

No clinical signs were observed that were related to the treatment. The mesenteric lymph nodes and the spleen of animals given Brewers Yeast increased significantly in weight both after four and eight weeks of treatment, as compared with the control group. In addition, in the group with the highest exposure, liver weights increased significantly after eight weeks of treatment. No macroscopic lesions were recorded. Microscopically, treatment-related changes were found in the liver (fatty change) and the mesenteric lymph nodes. In the mesenteric lymph nodes the changes consisted of diffuse moderate to marked granulomatous inflammation (clusters of large, pale staining –histiocytes) and focal/multifocal sinus dilation. In the mesenteric lymph nodes the increased weights corresponded to the severities and incidences seen microscopically.

2.1.4.3 Evaluation and main conclusions for the risk assessment

Here, a toxicity study of feeding with another single-cell protein – Brewers Yeast – is performed, followed by histochemical analysis of relevant organs The relevant organs were examined and histopathological changes in the lymph nodes were demonstrated with a diet containing 11 and 22% yeast. This study is relevant in that it confirmes that histological changes in draining mesenteric lymph nodes can be induced after feeding with another single-cell protein, but is of limited value as no further data intending to characterise the induced response is provided.

2.1.5 BioProtein and Brewers Yeast - One generation reproduction study in rat (Takawale, 2004a; Thestrup, 2004b)

The study was sponsored by Norferm and reported in a test report.

2.1.5.1 Study design

The study was designed to determine the effects of BP and Brewers Yeast on male and female reproductive performance, including gonadal function, oestrus cycle, mating behaviour, conception, pregnancy, parturition, lactation, weaning and on the growth and development of selected offspring up to the age of 12 weeks. One-hundred-and-twenty male and 120 female Wistar rats aged 5-6 weeks (males) or 8-9 weeks (females) at the start of the treatment were placed in five groups of 24 animals of each sex. The animals were treated orally by dietary administration of casein (Group 1, control), 6% BP (Group 2), 12% BP (Group3), 6% Brewers Yeast (Group 4) and 12% Brewers Yeast (Group 5). Males were treated 10 weeks before mating, during the mating and until necropsy after mating. Females were treated from

2 weeks before mating and until weaning the offspring on postnatal day 21. Selected offspring from each group (20 males and 20 females) were treated from weaning up to12 weeks of age with the diets from the respective groups. Blood samples were collected before necropsy and the relative content of different antibody isotypes (IgG1, IgG2a and IgA) reacting against BP was determined by ELISA. Three male and 3 female offspring (F1) from groups 1 and 3 (age 12 weeks) were immunised twice (14 days between) with BP in Freunds Incomplete Adjuvans by intraperitoneal injection. On day 24 after immunisation blood was collected before necropsy.

2.1.5.2 Observed effects

No treatment related clinical signs or changes in body weights were observed in the parents or offspring and no statistical differences were observed in the reproduction parameters or litter data. A dose-realted increase in absolute and relative weights of mesenteric lymph nodes in both sexes of parent animals were statistically significant in both BP groups and the 12% Brewers Yeast group. In the offspring, a correspondingly significant increase was observed only in the males receiving BP. A significant relative increase of liver weight was found in both males and females of the 12% Brewers Yeast group, as well as in the male offspring of the 12% Brewers Yeast group. No evident effect on liver weight was found in the parent or offspring fed BP. The relative spleen weights were increased in parent animals fed both levels of Brewers Yeast and 6% BP. In the offspring, both groups fed BP showed significantly increased spleen weights compared with controls. No treatment related macroscopic lesions were revealed in the parent animals or in the offspring in any of the groups. Microscopically, a dose related minimal to moderate increased focal/multifocal macrophage accumulation in the cortex and medulla of mesenteric lymph nodes in the BP treated groups were observed. The effect was more pronounced in male rats than in female rats. The effect was less prominent in the offspring than in the parents, and affected about 10% of the offspring exposed to BP. No histopathological changes were reported in the Brewers Yeast treated group.

The antibody response detected in BP fed animals was generally weak but statistically significant (1-3 fold). The offspring responded less than the parents. A similar or higher antibody level was detected in rats fed with Brewers Yeast, and the offspring rats responded less than parents. The responses were not dose related.

Following immunisation with BP, the titers of immunoglobulins were increased 1-2 orders in control rats compared to those in BP fed rats, and this was interpreted as tolerance induction.

2.1.5.3 Evaluation and main conclusion for the risk assessment

The histopathological examination confirms the data described in Mølck et al., 2002 (chapter 2.1.1). The well documented changes in the mesenteric lymph nodes indicate an intestinal response to the BP. An examination of the intestinal wall itself and the organised lymphoid tissue within the wall, the Peyer's patches were not conducted. Since the observed reaction in the intestine in the NABP treated animals may have been a response to the BP per se, a closer examination of the intestine and including the Peyer's patches could have added important information to the knowledge of the nature of the response to the BP. No histopathological changes were reported in the Brewers Yeast, which is in contrast with the findings described by Glerup *et al.* 2002 (chapter 2.1.4). The specificity of the detected humoral response is questioned, as similar levels of antibodies are found in some of the control samples. These results are also in contrast to the results described by Christensen *et al.* 2003 (chapter 2.1.2), where a specific and sustained induction of specific IgG2 antibodies was detected after feeding with BP.

2.1.6 BioProtein - Study in juvenile rats (Takawale, 2004b; Thestrup, 2004b)

The study was sponsored by Norferm and reported in a test report.

2.1.6.1 Study design

The study was designed to obtain information on the effect of BP on male and female juvenile rats. Twenty pregnant female SPF Wistar rats were used for the study. On postnatal day 21 (weaning time), 10 male and 10 female offspring were allocated to each of six groups and treated orally by dietary administration as follows: group 1: control (casein) diet to 12 weeks of age; group 2: 12% BP diet to 12 weeks; group 3: control diet to 6 weeks, then 12% BP to 12 weeks; group 4: control diet to 32 weeks; group 5: control diet to 6 weeks, then 12% BP to 32 weeks; group 6: control to 6 weeks, then 12% BP to 12 weeks, and control diet to 32 weeks. Before necropsy (groups 1-3 at 12 weeks of age, and for groups 4-6 at 32 weeks of age) blood samples were isolated for serological and haematological tests. The relative content of different antibody isotypes (IgG1, IgG2a and IgA) reacting against BP was determined by ELISA.

2.1.6.2 Observed effects

No treatment-related clinical signs were observed during the entire period of the study. The pathological examination included weighing of mesenteric lymph nodes, liver and spleen. Significant increases in mesenteric lymph node weight were seen at week 12 and 32 in BP dosed groups compared to the control groups. No gross pathological changes were observed among the dosed groups and the control groups. Microscopic findings were minimal to slightly increased macrophage accumulation, clusters of large pale-eosinofilic-staining cells in mesenteric lymph nodes of the female rats in Groups 2 and 3. No increased accumulation of macrophages was recorded in the mesenteric lymph nodes of males from Group 2 and 3 or in the mesenteric lymph nodes of the control group. In Group 5, 7 male and 10 female rats had minimal to moderately increased macrophage accumulation in the mesenteric lymph node. No macrophage accumulation was recorded in the mesenteric lymph nodes of males from Group 6 or in the mesenteric lymph nodes of the control group.

A significant increase in specific antibodies towards BP was seen. More specifically; a 15-fold increase in IgG2a and IgA antibodies was detected in the female rats, while IgG1-levels were less increased. For the male rats, a 22-fold increase in both IgG1 and IgG2a levels was found. A more profound increase in specific humoral response was found in this study, as compared to the findings in the one-generation study (chapter 2.1.5). For both female and male groups the specific antibodies seems to be present for a long time – more than 20 weeks after they have been fed BP.

The age of the rats when initially receiving BP is important with regards to the induction of the humoral response. Rats which received BP from week 3 of age responded with a significantly lower specific antibody titre than animals which were given BP from week 7, which was suggested to be due to induction of immunological tolerance when BP is given from an early age.

2.1.6.3 Evaluation and main conclusion for the risk assessment

The well documented changes in the mesenteric lymph nodes indicate an intestinal response to the BP. Also here, an examination of the intestinal wall itself and the organised lymphoid tissue within the wall, the Peyer's patches, could have added important information to the knowledge of the nature of the response ot the BP. The profile of the humoral response indicates the induction of a Th1 response, and supports the findings by Christensen *et al.* 2003 (chapter 2.1.2). The demonstration of the importance of age for the induction of a humoral

response is relevant, although the conclusion that this is a result of tolerance is speculative and not founded in the presented data.

2.1.7 Immune response in mice to ingested soya protein: antibody production, oral tolerance and maternal transfer (Christensen *et al., 2004)*

The study is published in *British Journal of Nutrition.*

This study is included as a basis for the BP assessment as also soya protein may constitute a foreign protein, and the Petitioner maintains that information on the immune response to soya protein may be useful for the assessement of BP.

2.1.7.1 Study design

The study was designed to describe the immune response in mice to soya protein including the evaluation of spontaneous antibody production, oral tolerance induction (detected as a suppressed antibody and cell-proliferation response upon immunisation with soya protein) and transfer of soya-specific immune components from mother to offspring. *Unprimed mice:* Age-matched F0, F1 and F2 male Balb/c mice were grouped upon weaning to15 mice/group. At age 6, 7, and 8 weeks blood samples were collected, mice sacrified and spleens subjected to cell proliferation assays. *Primed mice:* Age-matched F0, F1 and F2 male Balb/c mice were grouped upon weaning to 8-10 mice/group. At age 8 weeks, the mice were intraperitoneally immunised twice (14 days apart) with a soya extract mixture. *Mice with experimentally induced oral tolerance:* F0 and F2 male mice: upon weaning four groups of 8-10 mice /group. At 7 weeks one group from each generation was supplemented for 5 days with soya protein extract. After 14 days all the mice were immunised as above. *Transfer of mice at different ages between soya-containing and soya protein-free feed:* Seven groups (8-10 mice/group) were transferred from one feed to the other at different ages.

Antigen-specific antibody titre was determined by ELISA, and *in vitro* cell proliferative assay was performed using mice spleen cell suspensions cell.

2.1.7.2 Observed effects

Soya specific immune components were not found to be maternally transmitted, and F0-mice fed with soya developed a soya protein specific humoral response and oral tolerance against the protein. When this diet was replaced with a normal diet before mating – neither response was transferred to the F1-generation. However, ingestion of dietary soya protein during late pregnancy and lactation caused lasting antibody responses in the offspring, but without the induction of oral tolerance.

2.1.7.3 Evaluation and main conclusions for the risk assessment

The study is well designed and sheds interesting light on aspects of feeding with soya protein in general, but is considered as relevant in shedding light on the mechanisms behind the immune responses seen after feeding with BP.

2.2 Studies with pigs

The experiments include pigs with live-weight from 7 to 110 kg. The main objectives were to evaluate BP as a protein source for growing pigs by determining the effects on average daily gain (ADG), average daily feed intake (ADFI), feed efficiency (feed:gain ratio; F:G) and product qualities.

2.2.1 Bacterial protein grown on natural gas as feed for pigs (Øverland *et al., 2001)*

The study is published in *Acta Agriculturae Scandinavica.*

2.2.1.1 Study design

The paper includes three experiments, which were designed to determine the effect of adding increasing levels of BP up to 12% in conventional cereal-based diets on growth performance of weanling pigs and to evaluate the effect of adding BP to diets with normal and marginal protein levels on growth performance, carcass traits and sensory quality of backfat of growing-finishing pigs.

Experiment 1 was performed with 64 weaning pigs fed to satiation. Four treatments, including a control diet with no BP and three diets containing 4, 8 and 12% BP were evaluated. BP replaced crude protein from soybean meal, fish meal and bone meal. The experimental period lasted for 28 days (initial mean animal weight of 10.4 kg) and was conducted as a randomized complete block design with 16 pigs per treatment. Diets were balanced to meet the general amino acid requirement, and treatments were balanced to obtain the same dietary energy and crude protein content. Feed consumption and pigs' weight were measured weekly to determine ADG, ADFI and F:G ratio. In the diet with the highest BP 44% of the total dietary protein was from BP.

Experiment 2 was performed with 48 pigs fed restrictively (intial mean body weight 24.5 kg) to slaughter, and the objective was to evaluate BP inclusion in barley/soybean meal diets. The dietary treatments were arranged in a 2 x 3 factorial design with two crude protein levels (170 vs. 150 g/kg) and three levels of BP, replacing 0, 50% or 100% of the lysine in soybean meal with BP. In the high protein diets the BP content was 0, 5.2 and 10.7%. In the low protein diets the inclusion of BP was 0, 3 and 6.8%. In the diet with the highest BP inclusion, the percentage of crude protein from BP was 48% and 36% in the high and low protein diets, respectively. At both protein levels the calculated lysine content decreased with increased BP content in the diet. Diets were balanced to obtain the same dietary energy and crude protein content. However, in the low protein diets the highest proportion of BP had 6% lower crude protein content than the soybean meal diet.

Experiment 3 was performed with 24 pigs (intial mean body weight 27.8 kg) fed to slaughter. The dietary treatments consisted of a grain/soybean meal diet (control) and two diets containing 5.2 and 10.7% BP. In the two test diets 50 and 100% of the lysine were replaced by BP. The pigs were fed restrictively and diets were balanced to meet the animals' amino acid requirements, and treatments were balanced to contain the same dietary energy and crude protein content.

2.2.1.2 Observed effects

In experiment 1, the results showed that increasing the BP level up to 12% gave a linear increase ($P < 0.05$) in ADG. The highest level of BP resulted in a higher ($P< 0.05$) ADFI than the diet containing no BP. No differences in F:G was observed among treatments. No clinical deviation was observed as a result of exposure to BP in the diet.

In experiment 2, no protein level x protein source interactions were observed. Replacing 100% of lysine in soybean meal with BP reduced ($P < 0.05$) ADG during the first phase (0-7 weeks) of the growing period, although no differences ($P > 0.05$) in overall ADG was observed. The protein level did not significantly affect carcass traits, but there was an improvement in backfat firmness and colour.

In experiment 3, no significant differences were observed in ADG of pigs fed either BP diets or the soybean meal diet. No negative effects on the carcass were found but there was an improvement in backfat firmness and colour.

2.2.1.3 Evaluation and main conclusion for the risk assessment

In experiment 2, the reduction in ADG of pigs fed the highest BP level during the initial growth phase could be a result of the suboptimal lysine level in this diet and demonstrates the importance of balancing the dietary lysine content when BP is used as a protein source. This is also verified in Table 1 in the present assessment when comparing the amino acid content in BP, fish meal and soybean meal.

In experiment 3, lack of significant difference may be due to the limited number of animals used in this experiment. The BP diets showed lower lysine content than the soybean meal diet, although the differences was smaller than observed in experiment 2.

All experiments are of limited value for a risk assessment of BP.

2.2.2 Effect of bacterial protein meal grown on natural gas on growth performance and carcass traits of pigs (Øverland *et al., 2004)*

The study is published in *Italian Journal of Animal Science.*

2.2.2.1 Study design

The paper includes two experiments designed to 1) determine the effects of adding increasing levels of BP up to 12% in diet on growth performance and carcass traits of growing-finishing pigs, and 2) evaluate the effect of adding up to 15% BP to diets on growth performance and carcass traits of pig from weaning until slaughter.

Experiment 1 was performed with 18 pigs (initial weight of 26 kg) divided into three treatment groups. The dietary treatments consisted of a soybean meal diet (control) and two diets containing 6 and 12% BP. In the diet with the highest BP inclusion level, the percentage of crude protein from BP was 54%.

Experiment 2 was performed with 48 pigs with initial weight 11.4 kg. Four treatments were used, including a control diet with no BP and three diets containing 5, 10, and 15% BP. In the diet with highest BP level, 49% of the total dietary protein originated from BP.

2.2.2.2 Observed effects

No health related to the dietaty treatments encountered during the experiments.

Experiment 1:

Replacing amino acids from soybean meal with amino acids from BP reduced ADG and feed efficiency during the growing period, but no differences were seen in growth performance in the finishing period. For the overall period there was no significant difference ($P > 0.05$) in growth performance. Dietary analysis showed a lower total amino acid content and lower lysine content in the BP diets than the soybean meal diet. No significant effects were found on carcass traits.

Experiment 2:

In the period from weaning to five weeks post weaning there was a linear decrease ($P < 0.05$) in ADG with increasing BP level in the diet, and as similar effect was observed in the overall period from weaning until slaughter. Increased BP proportion increased ($P < 0.05$) backfat thickness and firmness.

Increasing the level of BP in the diet gave a reduction in the daily intake of total amino acids and lysine, which is probably the main reason for the reduced ADG observed at increased levels of dietary BP in these studies.

2.2.2.3 Evaluation and main conclusion for the risk assessment

From the results obtained in these experiments it is concluded that the addition of up to 12% BP in the diets for growing and finishing pigs had no adverse effect on overall weight gain. It is also suggested that BP can be used as a major protein source in diets for growing and finishing pigs. However, both of these studies were performed with limited number of animals and it is therefore difficult to verify this conclusion. Additional studies with high inclusion levels of BP are required to confirm the conclusions from these two studies.

The experiments indicate a sub-optimal lysine supply with a high proportion of BP in the diet which may explain the reduced growth performed. Thus, this may not be a negative effect of the protein source per se but demonstrates the importance of balancing the individual amino acids in the diet.

2.2.3 Effect of BioProtein on growth performance of fattening pigs (Sterten and Aglen, 2004; Landsverk, 2004a)

The study was conduced by the feed industry and reported confidentially.

2.2.3.1 Study design

The study was performed with 64 fattening pigs divided in two treatment groups. The experimental period was from an average body weight of 39 kg to slaughter weight (average 113 kg). The treatments consisted of a soybean/canola meal based control diet and a test diet with 6% BP. In the BP diet crude protein from the BP was balanced by a reduction in the level of protein from soybean meal. Diets were balanced both to meet the general amino acid requirement, and to contain the same dietary energy and crude protein content. The proportion of dietary crude protein from BP was 27% in the BP diet. The examination included histopathological examination of tissue samples from liver, kidney, spleen, thymus, ileum and distal jejunal lymph nodes from 15 pigs from each group.

2.2.3.2 Observed effects

No adverse effects in clinical health were observed related to the treatment. No significant differences ($P > 0.05$) were seen in ADG or F:G ratio.

There were no significant differences among groups in any of the organs examined histopathologically.

2.2.3.3 Evaluation and main conclusion for the risk assessment

The BP level used in the experiment was lower than the approved level. Only 15 fattening pigs were examined and histopathological examination was only conducted on a few tissues samples. No objective measurements such as registration of organ weight or enumeration of cell populations were included in the examination. No examination was performed on the systemic immune response (immunoglobulins, IgG, IgA) to the different diets.

2.2.4 Field trial with BioProtein in co-operation with Felleskjøpet Fôrutvikling; Bacterial protein meal as a feed ingredient for fattening pigs. (Karlengen, undated)

The study was sponsored by the feed industry and reported in a manuscript available from Norferm.

This study with fattening pigs was performed as a field trial at 15 commercial farms. The commercial concentrate mixture used contained 6% BP and there was no comparison with a control diet. Results were evaluated using a questionnaire to the farmers. This is not a scientific study, and therefore, it can not be used to evaluate the effect of BP on growth performance and feed efficiency in fattening pigs.

2.2.5 BioProtein in diets for piglets (Kløvstad, 2004; Landsverk, 2004b)

The study was conduced by the feed industry and reported in a manuscript available from Norferm.

2.2.5.1 Study design

The study was performed with 100 weaning pigs fed to satiation. The objective was to evaluate the effect of BP as a protein source in conventional cereal based diets on growth performance in weaned piglets. Two dietary treatments were used: 1) a control diet with fish meal as the control protein and 2) a test diet containing 9% BP. In the BP diet 35% of the dietary crude protein came from BP. The experimental period lasted 21 days and the average initial piglet weight was 12.2 kg. The experiment was conducted as a randomized complete block design with 50 pigs per treatment divided in two pens per treatment. Diets were balanced both to meet the general amino acid requirement, and to obtain the same dietary energy and crude protein content. Average daily gain was evaluated in weekly intervals from day 41 to 62.

The study included examinations for gross pathological and histopathological changes in 6 treated piglets and 6 control animals.

2.2.5.2 Observed effects

No treatment-related detrimental effects on clinical health were found.

No significant ($P > 0.05$) difference in ADG was observed in the separate periods, but the total gain (days 41-62) was higher ($P < 0.05$) in piglets fed the BP diet than in piglets fed the control diet. No differences were seen in feed intake or F:G ratio between treatments.

No significant differences were observed between the groups in any of the organs examined for histopathology (liver, lung, kidney, spleen, thymus, ileum and distal jejunal lymph nodes).

2.2.5.3 Evaluation and main conclusion for the risk assessment

The amino acid analysis of the diets showed a higher lysine, methionine and threonine content in the BP than in the control diet (7.7, 18.6 and 10.7%, respectively). The control diet showed a 11% higher cysteine content than the BP diet. No information concerning animal variation, i.e. standard error mean, is presented, and therefore, it is difficult to further evaluate the results. Because of the differences in amino acid composition between the diets it is also difficult to compare BP and the type of fish meal used as protein sources for piglets.

Only six treated animals were examined. No objective measurements such as registration of organ weight or enumeration of cell populations were included in the examination. Two

sections in the small intestine and one mesenteric lymph node (one section) were examined and this is not considered satisfactory for representative information on these tissues.

No examination was performed on the systemic immune response (immunoglobulins, IgG, IgA) to the treatments.

2.3 Studies with chickens

The main objectives of the chicken studies were to evaluate the effect of BP on: 1) ADG, feed intake feed efficiency and dressing percentage and 2) clinical health and target species safety.

2.3.1 The effect of bacterial protein grown on natural gas on growth performance and sensory quality of broiler chickens (Skrede *et al.,* 2003)

The study is published in *Canadian Journal of Animal Science.*

2.3.1.1 Study design

The paper includes three experiments designed to evaluate the effects of adding increasing levels of BP in conventional diets on growth performance of broiler chickens fed from day-old to slaughter at 35 days and sensory attributes of thigh meat after two months of frozen storage.

Experiment 1 consisted of six treatment groups, including a control diet with no BP and five diets containing 2, 4, 6, 8 and 10% BP. BP replaced crude protein from soybean meal. In the highest BP diet 33% of total crude protein was of BP origin. Diets were balanced both to meet the amino acid requirement of the animals, and to obtain the same dietary energy and crude protein content. A total of 720 chickens were used and the experiment was designed using 48 pens with 15 chickens in each, giving eight replicate pens per treatment. The chickens were weighed and feed consumption was registered penwise at start, day 14 and before slaughtering at 35 days of age.

In experiment 2, chickens were fed diets containing 0, 3, 6 and 9% BP at two protein levels (231 and 209 g/kg). The high protein diets were formulated to meet or exceed crude protein and all amino acid requirements for broiler chickens. The low protein diets were formulated to meet the crude protein and amino acid requirements in the 3-5 week period, but provided suboptimal levels of crude protein, methionine + cysteine and threonine in the 0-3 week period. A total of 1024 chickens were used and the study comprised of 64 pens, each with 16 chickens, distributed with eight replicates per treatment.

The 3rd experiment was performed as a follow-up study using the four high protein diets from experiment 2. Each treatment had two replicate pens, each with 15 chickens per treatment.

2.3.1.2 Observed effects

Experiment 1: Levels of BP up to 6% showed no effect on 0-5 week gain, while 8 and 10% BP reduced gain, especially during the first two weeks of the experiment. No negative effects were found on sensory analyses of meat.

The results obtained in experiment 2 were similar to those observed in experiment 1. Independent of dietary crude protein level, the highest proportion of BP reduced growth, whereas growth responses to the lower levels of BP were similar to the control diet which consisted of soybean meal as the main protein source. In the period 14-35 days, feed intake in chickens fed 3, 6 and 9% BP was lower ($P < 0.05$) than those fed diets with no BP. This resulted in an overall feed conversion rate that was better for the birds fed 3 and 9% BP than

those fed no BP. Some of the chickens showed signs of the "Malabsorption syndrome", however, the frequency was not related to the BP level.

In experiment 3, no differences ($P > 0.05$) in body weight or feed conversions were observed between treatments. This means that in this experiment no negative effect on growth performance was observed when chickens were fed diets containing 9% BP.

2.3.1.3 Evaluation and main conclusion for the risk assessment

Experiment 1: One weakness with this study was that dietary amino acid content was not analysed but based on tabulated values. Thus, it is difficult to evaluate the negative effect on growth performance obtained for the two highest BP levels.

From experiment 1 and 2 it is concluded that diets containing 6% BP resulted in growth performance similar to those obtained with soybean meal, and that a higher level caused reduced gain. The authors' claim that it was not possible to use regression analysis to evaluate of optimal growth performance, however, they don't describe or discuss why this was not possible.

In these studies BP and soybean meal were compared as protein supplements in diets for chickens, however, the studies are of limited value for a risk assessment of BP.

2.3.2 Broiler chicken trial with BioProtein (Faaland Schøyen *et al.*, Undated)

The study is reported in a manuscript available from Norferm.

2.3.2.1 Study design

The study was performed to determine the effect of BP on weight gain, feed intake, feed efficiency, dressing percentage, clinical health and target species safety. The experiment was carried out with 120 chickens, which were randomly allocated into two dietary treatment groups (four pens per treatment with 15 chickens per pen).The dietary treatments were a control diet with no BP and a test diet where soybean meal was replaced by BP at a level of 6%. The chickens were weighed and feed consumption was registered per pen at the beginning of the experimental period, followed by weekly intervals and at the end of the experimental period. All birds were slaughtered at day 36 and twelve birds per treatment were used for determining slaughter weight and dressing percentage. Examination for gross pathological and histopathological changes was performed on chickens that died during the experiment and on slaughtered chickens. The examination included 12 slaughtered chickens in the treatment group and 12 control animals. Histopathological examination included tissue samples from lung, liver, kidneys, heart, jejunum, spleen and *Bursa fabricii*.

2.3.2.2 Observed effects

Average weight gain per pen from 0 to 14 days was higher ($P < 0.02$) for chickens fed the BP diet compared to the control chickens. No differences in feed conversion ratio between treatments were observed in this period. From day 14 to 36, no differences in growth performance or feed conversion rate was observed. However, when evaluating the overall growth response from day 0 to 36, chickens fed the BP diet had a higher ($P < 0.05$) weight gain and a better ($P < 0.05$) feed conversion rate than the control chickens.

The pathological examination revealed no apparent differences between the chickens fed BP diet and the control diet. Variations in the morphology observed were considered to be within a normal range.

2.3.2.3 Evaluation and main conclusion for the risk assessment

Only one chicken feeding trial is not sufficient to document that the BP has no pathological effect on the chickens, and higher doses should have been used.

The examination did not include any objective measurements such as weight of organs or enumeration of cell populations. Only one location in the proximal jejunum was examined. Chickens do not have lymph nodes, and to compensate for lack of investigation of lymph nodes, examination of intestinal wall containing organised lymphoid tissue such as the caudal small intestine and caecum (caecal tonsillar tissue) could have contributed information about a potential response in the intestinal lymphoid tissue.

No examination was performed on the systemic immune response (immunoglobulins, IgG, IgA) in chicken exposed to BP.

2.4 Studies with ruminants

BP has been approved since 1995 for use up to 8% in feed for veal calves from 80 kg live weight.

No studies documenting the efficacy and safety of BP for veal calves exist although studies of other target animals are available.

2.5 Studies with pets and fur animals

2.5.1 Evaluation on nutritional quality of BioProtein in wet cat standard product for cat. (Lavanant, undated)

The study was conduced by the feed industry and reported confidentially.

2.5.1.1 Study design

Cats were fed a control diet or a trial diet with 2.5% BP for 1 month. Food consumption, body weight and faecal quality were measured. The number of animals included or their ages are not reported.

2.5.1.2 Observed effects

The food consumption, body weight, and faecal consistency in cats fed the diet containing BP did not differ from values for the control animals.

2.5.1.3 Evaluation and main conclusion for the risk assessment

The report is insufficient, and has no value for a risk assessment of BP.

2.5.2 BioProtein – An 8- week local tolerance study after oral dosing in the cat. (Glerup, 2001)

The study was sponsored by Norferm and reported in a test report.

2.5.2.1 Study design

This study aimed to assess the tolerance of BP in cats, after oral treatment for 8 weeks. Cats were selected as the experimental animal due to the future potential of this being a target species. Four groups of domestic cats (3 males and 3 females per group) were fed with 0%

(group 1), 5% (group 2), 10% (group 3) and 20% (group 4) BP for 8 weeks. Clinical signs and food consumption were recorded daily, and body weight recorded weekly. Blood samples were taken shortly before necroscopy for antibody analyses, however, the results were not provided. The animals were subjected to macroscopic pathological examination. Mesenteric lymph node weights were recorded and representative specimens sampled for later histopathological studies, however the results were not provided.

2.5.2.2 Observed effects

No treatment-related macroscopic changes were observed in the cranial-, thoracic-, abdominal cavities or gastrointestinal tract. No treatment-related clinical signs were seen during the study. Growth rates were unaffected by the inclusion of BP in the diet in all groups except for females in group 4, where a significantly lower body weight was seen on day 43 (a difference that was considered incidental and not treatment-related). A tendency of absolute and relative increase in weight of the mesenteric lymph nodes was seen for BP-treated groups compared to the control, and this increase was statistically significant in the females of group 4.

2.5.2.3 Evaluation and main conclusion for the risk assessment

The results presented in this report are preliminary. An enlargement of mesenteric lymph nodes after feeding with BP in an intended target animal is described, while the nature of this effect was not investigated. The reduction of the body weight of the females in group 4 was interpreted by the author as incidental and not treatment related, although a statistically significant increase in mesenteric lymph node weight was only found in this group.

2.5.3 Bacterial protein grown on natural gas ("BioProtein") as feed for the carnivorous blue fox (*Alopex lagopus*) (Skrede & Ahlstrøm, 2001; Skrede & Ahlstrøm, 2002)

The study is spornsored by Norferm and reported in a manuscript (Skrede & Ahlstrøm, 2001) available from Norferm, and also published in *Journal of Nutrition* (Skrede & Ahlstrøm, 2002). The manuscript and the scientific paper describe the same study but the scientific paper does not include the effect on mesenteric lymph nodes in BP exposed animals.

2.5.3.1 Study design

The study was designed to examine the suitability of BP as a protein source in diets for carnivores. The diets were formulated to satisfy the nutritional requirements of dogs, however foxes were used as model animals, based on a previous report that blue foxes and dogs have very similar digestive capacity (Ahlstrøm & Skrede, 1998). Four dog diets containing 0 (control group), 4, 8 and 12% BP were used. The diets contained comparable levels of crude protein and covered the minimum requirements for essential amino acids.

Each group consisted of 10 weaned male and 10 weaned female blue fox cubs (approximately 2 months old with a weight of approximately 2.8 kg). The animals received the diets for four months. Data on body weight and feed consumption were regularly recorded. Mesenteric lymph nodes from six males in each group were removed and weighed immedially after sacrifice.

In addition, a 7 days experiment on the digestibility of the diets was also examined.

2.5.3.2 Observed effects

All animals accepted the diets. There were no signs of health problems and no mortality occurred during the experiment. Evaluation of fur quality showed no significant differences,

and there were no significant differences in diets in the digestibility of protein, fat or carbohydrate among the treatments.

The weight of the mesenteric lymph nodes increased with increasing BP concentration in the diet.

2.5.3.3 Evaluation and main conclusion for the risk assessment

The results are of value for blue fox but for evaluation of the effect of BP in dogs, studies on dogs are required. The trial only included young animals, and the effects of BP on pregnant or lactating females and adults should have been examined.

The weight of the mesenteric lymph nodes was studied in selected male foxes only. The lymph nodes of female foxes should also have been weighed (the lymph nodes of the female cats were more affected than in males) and the examination of lymphoid tissue (GALT) should have been more extensive.

No immunological examination, such as measurements of immunoglobulins has been reported.

2.6 Studies with salmon

Few experiments have been conducted on health of fish fed BP.

2.6.1 Bacterial protein as a protein source in diets for Atlantic salmon (*Salmo salar*) (Øverland, undated; also presented in short form by Aas *et al.,* 2004*)*

The study is reported in a manuscript available from Norferm and in a short version in an abstract to an international symposium.

2.6.1.1 Study design

The study was designed to investigate BP as a protein source for Atlantic salmon in terms of growth, feed utilization, energy budget and nitrogen metabolism. BP constituted 4.5, 9, 18 and 36% of the diets and was compared to a fish meal based diet without any BP added. The average initial body weight was 170g in a 8 weeks exposure period, followed by 1 week fasting to allow heat increment estimation. Each diet was fed in triplicate tanks with 18 fish per tank.

The diets were made iso-N and iso energetic, but were not balanced, with regards to AAs since it was claimed that the AAs were equal to fish meal with the exception of higher tryptophane and threonine content and lower lysine content Skrede *et al*. (1998).

2.6.1.2 Observed effects

No mortality or health problems related to the experimental diets tested were observed, but some cataracts occurred which did not appear to be related to the BP exposure. A dose dependent improvement in growth and feed utilisation were found, and a significant improvement in energy retention and nitrogen retention in fish on the three highest BP levels. The concentration of urea in liver and plasma increased with BP level although only the control and the two highest BP exposure groups were measured.

2.6.1.3 Evaluation and main conclusion for the risk assessment

This growth study was conducted for 8 weeks, which is a shorter period than usually used for growth experiment (12 weeks). The report gives the mean values from triplicate tanks without giving any information regarding deviation from the means. No investigations were performed on immunological endpoints or on lymphoid tissue. It was only claimed that no

clinical symptoms were observed, however there was no description on which clinical symptoms were examined. Although fish have a different lymphatic system than mammals, at least the blood, liver, head kidney, spleen, and the GI-tract should have been examined. The dietary AAs were not balanced which are known to affect both feed intake and growth (Witte *et al*., 2000; Vieira *et al.,* 2004; Wijtten *et al.,* 2004; Carew *et al.,* 2005).

More recently Storebakken *et al*., (2004) and Berge *et al*., (2005) have published results on effects of feeding BP to salmon for longer periods of time, 1 year in fresh water and 5 months in saltwater, respectively.

2.6.2 Bacterial protein grown on natural gas in diets for Atlantic salmon, *Salmo salar*, in freshwater (Storebakken *et al*., 2004)

The study is published in *Aquaculture.*

2.6.2.1 Study design

The study was designed to assess the effects of replacement of protein from fish meal on nutrient digestibility, growth, body composition, survival or histopathological indices in the fish. The AAs in fish meal were replaced by 0, 6.25, 12.5, 25 and 50% AAs from BP, corresponding to respectively 0, 5, 9.9, 19.3 and 37% BP of the diet. Triplicate tanks were used and the initial body weight was 0.2 grams. 600 fry were distributed to each of 15 tanks. The fish were fed the diets for a period of 1 year. Histological examinations were conducted on liver tissues and intestine as well as a cross section of the carcass (muscle, skin and kidney) from 20 fish fed the highest and lowest inclusion level of BP, i.e the 6.25 and 50% amino acid replacement, but not on the control fed group.

2.6.2.2 Observed effects

The growth rate was significantly depressed in fish fed the two highest levels of BP during the first 253 days, and in the highest dosage group during the whole experimental period. The survival was significantly lower in the fish fed the highest BP level compared to the controls. The whole body lipid deposition and the relative liver weight increased with increasing BP level in a curvilinear manner. No histopathological effects were revealed.

2.6.2.3 Evaluation and main conclusion for the risk assessment

The hepatic lipid deposition and increased liver weight could be a result AA imbalance, but it is unclear whether dietary AAs were balanced. The authors conclude that 25% of the AAs (e.i. 19.3% of the diet as BP) may be an optimal level of BP, but at that level depressed growth rate and increased lipid accumulation were found. Histological examination of the control fish should have been included; also immunological reactions should have been investigated.

2.6.3 Bacterial protein grown on natural gas in diets for Atlantic salmon, *Salmo salar*, in saltwater (Berge *et al.*, 2005)

The study is published in *Aquaculture.*

2.6.3.1 Study design

The study was designed to evaluate the effects of partial substitution of fish meal with BP on growth, feed conversion ratio, health, and product quality of salmon raised at a semi-commercial scale in seawater net-pens. Diets replacing 0, 10 or 20% fish meal with BP (0,

17.2 and 33.1% of the dietary protein) were tested. Duplicate groups of salmon (1.4 kg body weight) were fed the diets for 5 months. Each pen contained 1000 salmon. At the end of the feeding experiment, 5 fish per pen was used for histopathological evaluation of the distal intestine, and haematocrit values were also analysed in the same fish. Additionally 5 fish per pen was analysed for colour (colour card, carotenoid concentration) and 3 fish per pen collected for sensory analyses.

2.6.3.2 Observed effects

No significant differences were observed in growth rate, dressed-out carcass percentages, liver or intestine to body wt percentages among treatments, but the growth rate tended (not statistically significant) to decrease with increased BP inclusion in the diet. Carcass and visceral fat contents decreased with increased levels of BP, but the quality of the fish was unaffected. Histological examination did not show abnormalities or sign of reactions against BP, except that one fish fed 10% BP was severely inflamed, heavily infiltrated with leucocytes and did not contain any absorptive vacuoles. This fish had a normal body weight and haematocrit, so it was concluded that the inflammation was not related to BP exposure. No significant differences were found in hematocrit values among the treatments.

2.6.3.3 Evaluation and main conclusion for the risk assessment

BP inclusion up to 33% of the diet is approved for salmon in seawater however the study contained a maximum of 20% BP. The authors claim that the AAs in BP is similar to that in fish meal referring to Skrede *et al.* (1998), however levels of certain AAs differ considerably between fish meal and BP (Table 1). The diets did not seem to be analysed for their AA composition despite the fact that deviations in diet AA may affect both the feed intake as well as growth.

Only the intestine was sampled for histological examination in this study, however since immunological or lymphoid tissue reactions have been demonstrated in other species following BP exposure tissues as headkidney, liver, spleen and the blood should have been examined.

3 Residues of BioProtein compounds in animal products

BP consists of the common nutrients in animal feedingstuffs although the composition deviates to some extent from other common feedingstuffs. The deviating amino acid profile in feedingstuffs based on BP will, however have little or no effect on the total amino acid composition in the animal products as this can be adjusted for according to the animal´s need.

The elevated levels of phospholipids in BP contains a narrow compostion of common fatty acids more typical animalian than vegetabilian products, and feeding may influence the firmness and color of the animal fat. The relatively high level of lipopolysaccarides in BP will most likely be metabolized and is not expected to influence on the animal product.

The majority of nucleic acids present in BP are degraded in the intestine of the animal, although traces of the nucleic acids from BP may still present in animal products.

The relatively high concentrations of copper in BP and iron in NABP may also result in increased levels in animal products, particularly in livers.

Development of particular undesirable substances during the production process is not documented and residues of process generated foreign compounds in the animal products are not likely to occur.

4 Risk characterization

4.1 Animal safety

The studies have been assessed in an attempt to determine whether the nature of the immune stimulatory effects first described in laboratory rats after feeding with nucleic acid reduced BP (NABP) is sufficiently explained. In retrospect, a similar response was seen after feeding untreated BP. Most of the concern regarding the side effects of BP in feed is related to this immune response. The main findings included changes in weight and morphology of mesenteric lymph nodes, followed by induction of specific antibodies (Mølch *et al*., 2002; Christensen *et al*., 2003). Histopathological examination after feeding with NABP also revealed changes in the intestines and several internal organs indicating systemic effects (Mølch *et al*., 2002). The changes in the intestine comprised accumulation of eosinophilic granulocytes and pigment containing cells. These findings could be related to the high iron content in this product, but an irritation/inflammation induced by the NABP per se would be more likely. The presence of specific IgM and IgG antibodies supports the latter.

The Petitioner claims that the immune response seen in BP-fed mice/rats is most likely a normal response to ingestion of large doses of a foreign antigen, and further, that oral tolerance towards this protein is induced over time. However, these interpretations are not adequately supported in the supplied documentation. That feeding with Brewers Yeast also results in immune-stimulatory effects on draining mesenteric lymph nodes, does not provide any further evidence on the detailed mechanisms behind the immune responses seen after feeding with BP. A tendency towards adaptation might be indicated in some of the studies as less effects on lymphoid tissue were found in offspring than in the parents fed on BP, rats receiving BP from 3 weeks of age responded with a significant lower specific antibody titre than animals receiving BP from 7 weeks age, and the immunoglobulin titers as a response to immunization with BP (injection) were lower in rats fed BP than in control rats (Clausing & Bøgh, 2002; Takawale 2004a; Takawale, 2004b; Thestrup, 2004b). However, results from two experiments on mouse/rat (Christensen *et al*., 2003; Thestrup, 2004b) argue against tolerance induction as BP feeding induced sustained specific humoral responses of the IgG2a isotype, indicating a bias towards a Th1 response. Tolerance induction is normally favoured by a bias towards a Th2 response.

Which of the main contents of BP (phospholipids, lipopolysaccarides, nucleic acids or the protein structure, or combination of these compounds) that is responsible for the immuno-logical changes observed, has not been fully determined. Evidence has been presented showing that nucleic acid-reduction of BP does not influence oral immunogenicity, as NABP and BP induced similar specific immune responses (inducing identical IgG1 and IgG2a-responses) (Christensen *et al*., 2003). However, the particulate structure of BP has been shown to influence the observed immune response (Christensen *et al.*, 2003) as the systemic immune response was avoided by ingestion of BP free of whole cells. This suggests a central role for either the phospholipids and/or lipopolysaccharides together with the protein in immune response triggering.

The studies conducted in target species: pig, chicken, cat, fox and salmon, have not included adequate examinations of the immune effects from ingestion of BP. In particular, the possibly adverse effects due to exposure to foreign antigens early in life remain to be adequately studied. In pigs, lymphoid tissue was examined in two studies with low levels of BP: a study on fattening pigs with 6% BP, and in a short term study of piglets (21 days) with 9% BP

(Sterten & Aglen, 2004; Kløvstad, 2004). No histopathological effects were observed in the pig studies. In chicken no immunopathlogical effects were found at the dietary level of 6% (Faaland Schøyen *et al*., undated). In cats and foxes fed BP increased mesenteric lymph nodes were found (significanty increased in cats fed 20% and in fox fed 12% BP in the diet) (Glerup, 2001; Skrede & Ahlstrøm, 2001). Restricted histopathological examinations (intestine, liver) of salmon fed BP did not reveal effects.

In the remaining target animal studies the main focus has been on production parameters; weight gain, feed intake, feed efficiency, observation of clinical health, and product qualities. When the contents of amino acids were balanced, the inclusion of low levels of BP (up to 9%) tended to stimulate the gain of growing pigs (Øverland *et al*., 2001; Kløvstad, 2004), and the same tendency was found in chicken with up to 6% BP (Faaland Schøyen *et al*., undated). Higher feed levels of BP increased the risk of the opposite effect.

In salmon, a dose dependent improvement of growth was reported from the short term experiment (8 weeks) up to 36% BP in the diet (Øverland, undated, Aas et al., 2004). However, in a longer term experiments with salmon, depressed growth and increased liver weight were observed in freshwater at 19% BP and a tendency of reduced growth in saltwater at 20% BP (Storebakken *et al*., 2004; Berge *et al*., 2005). These results indicate effects at BP levels considerably lower than those currently approved (19 and 33%, respectively, in feed for salmon in fresh and saltwater, respectively).

In the supplementary dossier from Norferm AS it is claimed that the principal application of BP is in the feed to Atlantic salmon. Further the Petitioner claims that BP has been extensively used in salmon feed at levels up to the maximum permitted and that there have been no reported reactions in consumers or on the quality of the salmon produced with feed containing BP. However, very few studies have been published/provided to document the claimed lack of effects of BP in salmon. Digestibility has been examined, however clinical, biochemical and immunological effects of BP in fish diets have not been sufficiently evaluated in salmon.

As effects in salmon are demonstrated at BP levels considerably lower than those currently approved, it can be argued that the inclusion levels of BP should be reduced. The basic documentation for the approved dietary level of BP to salmon, calves and pigs in 1995 may have been very restricted.

Until possible effects in the immune system have been satisfactorily examined an inclusion level of 6% BP in the diet to salmon and to terrestrial target animals would reduce the risk of potentially adverse effects in the animals.

4.2 Consumer safety

Consumption of products from animals fed moderate levels of BP is not considered to constitute any human health risk with regard to the somewhat changed compostion, compared to other common feedingstuffs in terms of amino acids, phospholipids and lipopolysaccarides.

The high levels of nucleic acids present in BP might leave small amounts of bacterial nucleic acids in the animal products. However, transfer of nucleic acids from animal feed to humans has not been documented. The possibility of increased levels of nucleosides and DNA in human food could theoretically be of relevance for people with gout disease and the adjuvant effect of bacterial DNA might constitute an increased risk for food allergy.

The elevated copper concentrations in BP may cause increased concentration in certain animal products, particularly in the livers. This mineral is essential for animals and humans,

but the total intake should be restricted. A contribution to the dietary intake of copper mineral from BP is not expected to constitute a problem.

There is no evidence of development of any undesirable substances during the production process, which could represent a human health risk via residues in animal products.

The risk associated with the human consumption of products from animals fed on BP is considered negligible. However, the production of single cell protein for feed production represents a relatively new scientific approach which implies precautionary handling.

5 Conclusions

5.1 Animal safety

- BP inclusion in animal diets induces a dose-dependent immune response in several species (rodents, fox, cat). However, the target animal studies were not satisfactorily designed to reveal effects on the immune system.
- The most prominent responses observed after feeding with BP are histopathological changes in the draining mesenteric lymph nodes and induction of specific antibodies.
- Effects in animals after feeding early in life, when the animal´s immune system is not fully developed, have not been sufficiently determined, particularly in target species.
- In rodents, feeding of BP early in life tends to induce lower humoral responses than in older animals, indicating the development of adaption to the protein. However, the induction of antibodies of the IgG2a isotype, indicates the induction of a Th1-response which argues against the induction of oral tolerance.
- By feeding mice with whole-cell free preparations, the systemic immune responses to BP were avoided indicating that the particulate structure influences the observed immune response.
- Low dietary levels of BP do not seem to produce any adverse effects but may have a stimulating effect as the body weight gain was increased at 9 and 6% BP in pigs and chicken, respectively.
- The currently approved inclusion rates of BP in animal feed appear to be high since clinical, biochemical and/or immunological effects have been observed in target animals at approved levels.
- Until possible effects in the immune system have been satisfactorily examined an inclusion level of 6% BP in the diet to salmon and to terrestrial target animals would reduce the risk of potentially adverse effects in the animals.
- Most of the existing documentations on BP are unpublished manuscripts or internal test reports. Studies published in peer-reviewed journals imply a quality assurance of the data which is important for a powerful risk assessment.

5.2 Consumer safety

The risk to consumers of products from animals fed low levels of BP is regarded as negligible.

6 Gaps in knowledge

The most serious concern for using BP in animal feed is its effect on the immune system discovered in certain animal species. One concern is whether this could affect the animal´s ability to handle additional stress situations, such as infection with pathogenic microorganisms. To reveal whether the immune system has changed its capacity to handle regular infections that are common during the life cycle, it is suggested that challenge studies should be performed, using pathogenic microorganisms in animals fed various doses of BP. The effect on resistance to diseases may principally be negative or positive, and expected to be dependent on dietary level.

Investigations of representative samples of relevant lymphoid tissues for histopathological examinations and humoral responses in target species should be conducted.

The rat trials with the ordinary BP product did not include an examination of the intestine. A close examination of the intestine and including the Peyer's patches could have revealed important information on the nature of the response to BP since the reaction observed in the intestine in the NABP treated animals could have been a response to the BP per se. However it is important to have in mind that histolological examination alone is not sufficient to determine the type of immune reaction taking place.

Further experiments should be conducted to characterise the nature of the response seen in laboratory animals and some of the target animals. The rat/mouse model may not be representative for all the target animals, but it is a suitable model system for characterising underlying mechanisms as all the tools needed for future studies are available.

Before an extention of the approval to other animal categories, chronic feeding experiments should be conducted, including clinical, subclinical and immunological examinations in these species.

Further investigations should be conducted on which of the BP components (phospholipids, lipopolysaccarides, nucleic acids or the protein structure), or compounds altogether that are responsible for the immunological changes.

ASSESSED BY

Panel of animal feed

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