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(54) Title: METHOD FOR IMPROVING THE FERTILITY OF ANIMALS

(57) Abstract: This invention is in the field of animal food, in particular food for female animals in their reproductive phase. Food according to the invention improves the fertility of animals and increases their chances on a pregnancy. It was found that the fertility of animals may be improved by feeding the animal with a certain amount of naturally occurring immune enhancing ingredients such as beta-glucans and/or phytohormones like auxin or gibberellic acid. Also, combinations of these substances, in particular the combination of beta-glucans and phytohormones, were shown to have a synergistic effect in that they improved the fertility of the animal more than these individual components on their own.

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METHOD FOR IMPROVING THE FERTILITY OF ANIMALS

This invention is in the field of animal food, in particular food for female animals in their reproductive phase. Food according to the invention improves the fertility of animals and increases their chances on a pregnancy.

A wealth of literature has been published on the treatment of infertility in man and animals. Reproductive medicine has revealed a great number of treatments that have good success rates for many different indications.

Clomiphene citrate is a commonly prescribed fertility drug. It stimulates the brain to release luteinizing hormone, which causes egg maturation and release from the ovary. The drug is taken for just a few days and then stopped. A brief fertile period may follow. A drawback is that more than one egg may be released, resulting in a multiple pregnancy. In addition, nearly half of all pregnancies achieved in this way result in spontaneous abortion. Perhaps the miscarriage rate is so high because such medications only force ovulation without correcting underlying deficiencies or hormonal abnormalities.

Botanical medicines on the other hand, nourish and support the female reproductive system to restore hormonal balance. Many dietary supplements have been described that function in improving the fertility of man and animals.

In order to improve reproductive health of male and female animals it has been recommended to feed a good multivitamin preparation and include sufficient amounts of supplements in the diet, such as zinc, vitamin E and the amino acids arginine and taurine.

Because semen contains high concentrations of zinc, supplementing the diet with 30-60 mg zinc a day may improve sperm count and sexual function. A good dietary source of zinc is pumpkin seeds, long recommended as a fertility-enhancing food and a remedy for benign prostate enlargement. Pumpkin seeds also are rich in two other nutrients beneficial to male sexual functioning: the plant steroid beta-sitosterol (which binds to the testosterone receptor) and vitamin E.

Vitamin E has previously been labeled the anti-sterility vitamin. Vitamin E is crucial to proper reproductive function in both men and women. In fact, the chemical name for vitamin E, "tocopherol," originated from the Greek words tokos, which means "offspring," and phero, which means "to bear." One of the body's main antioxidant nutrients, vitamin E protects hormones from oxidation. As vitamin E becomes less available in processed foods and exposure to harmful oxidizing agents increases, supplementation with 400-800 IU a day may improve fertility for some men.

Arginine has been shown to raise sperm counts and sperm motility. The recommended dosage is 2-4 g a day. Taurine, another amino acid that may enhance sperm production and motility, is supplemented at 2-4 g a day.

The following herbs are often recommended by naturopathic doctors and herbalists to help restore female fertility.

Chaste tree berry (*Vitex agnus-castus*): This herb stimulates the release of luteinizing hormone (LH) from the pituitary gland in the brain. This, in turn, promotes ovulation. Chaste tree berry may restore normal periods in women with amenorrhea (lack of menstrual periods).

Dong quai (*Angelica sinensis*): Widely used for female complaints, including menstrual irregularities and infertility, dong quai can tone a weak uterus by promoting metabolism within the organ, regulating hormonal control and improving the rhythm of the menstrual cycle.

Red clover blossoms (*Trifolium pratense*): These beautiful little flowers are categorized in many herbals as fertility promoters. Chemical analysis shows that the herb is rich in coumestans and isoflavones, estrogenlike compounds that may promote fertility, particularly in women who are deficient in estrogen.

Licorice (*Glycyrrhiza glabra*): This plant contains hormonally active compounds categorized as saponins. A Japanese study found licorice-based medicines improved menstruation in women with infrequent periods. The study also found that licorice helped women with elevated testosterone and low estrogen levels, as commonly occurs in polycystic ovary disease.

Siberian ginseng (*Eleutherococcus senticosus*): This and other tonic botanicals can improve fertility by enhancing overall health and vitality. Siberian ginseng also acts on the brain to promote regulation of reproductive hormones.

Despite all these products that may improve the fertility of animals, there is still room for alternative products that can play a role in this process.

Surprisingly, it has now been found that the fertility of animals may be improved by feeding the animal with a certain amount of naturally occurring immune enhancing ingredients such as beta-glucans and/or phytohormones like auxin or gibberellic acid. Also, combinations of these substances, in particular the combination of beta-glucans and phytohormones, were shown to have a synergistic effect in that they improved the fertility of the animal, even more than these individual components on their own.

The invention therefore relates to the use of an animal feed comprising

between 0.05 and 500 milligram of beta-glucans per kilogram of feed and/or between 0.1 and 1000 milligram of phytohormones per kilogram of feed for improving the fertility of animals.

The term infertile animals or animals with impaired fertility is used herein to refer to animal populations that have difficulties in getting pregnant. In particular however, the term is used to indicate individual animals that have difficulties in getting pregnant. The invention proved particularly useful for getting animals into heat earlier, so that they produced offspring earlier resulting in an increased number of offspring of the total population. It was also observed that the number of newborns per parent animal increased by using the method according to the invention. This is particularly useful in commercial stock farming where even small increases in the productivity of the stock may contribute significantly to the commercial profits.

Improving the fertility of animals as used herein also relates to increasing the number and/or quality of the offspring of animals, in particular vertebrate animals, more in particular domestic animals such as farm animals. Farm animals are animals that are kept for profit, such as for instance animals selected from the group consisting of cattle such as cows, pigs, horses, rabbits, deer, ostrich or fur-animals like mink. The term animals as used herein is particularly intended to exclude humans.

The causes of reduced fertility or even infertility are not yet completely understood. Even in animal populations that are completely germ-free such phenomena exist. The effects in this study were observed in an otherwise healthy population. Mortality rates in the control group were the same as the mortality rates in the group that received the feed according to the invention.

Phytohormones are herein defined as molecules that function to coordinate plant growth and development. The compounds that have been considered as plant hormones are for instance: indole-3-acetic acid (auxin), cytokinin, gibberellin, ethylene, abscisic acid. In addition, brassinosteroids, jasmonic acid and salicylic acid have been shown to have important growth regulating activities and are considered to function as phytohormones.

The invention therefore particularly relates to a method wherein the phytohormone is selected from the group consisting of indole-3-acetic acid (IAA, auxin), cytokinin, gibberellin, gibberellic acid, ethylene, abscisic acid, brassinosteroids, jasmonic acid and salicylic acid.

Particularly good results were obtained when the animal feed was supplemented with free IAA instead of conjugated IAA. Free IAA and conjugated IAA are

known compounds. Free IAA is a naturally-occurring plant growth phytohormone which has been extensively studied. In plants, most of the IAA occurs in a conjugated form (Slovin et al. 1999, *Biochemistry and molecular biology of plant hormones*, Elsevier, Amsterdam. P115-140), either conjugated to sugars via ester linkages or to amino acids and peptides via amide linkages.

Free IAA is readily available as a commercial product. It may be synthesised chemically or prepared in a biological way. IAA producing micro-organisms are widespread in nature. Yeast, fungi and many bacteria as well as plants are known to convert precursors of IAA into free IAA. In addition to the L-tryptophan conversion by bacteria, also L-tryptophan independent biochemical routes towards free IAA are described extensively (J. *Plant Growth Regul* (2001) 20: 198-216).

A well known bacterium, capable of producing free IAA is *Azospirillum Brasilense* (AB). At the end of the growth phase in a regular fermentation process, AB is able to convert L-tryptophan into free IAA. To increase the efficiency of this conversion, a small amount of synthetic free IAA may be added to the media. Via a feedback mechanism, AB increases the conversion of L-tryptophan into free IAA.

Final concentrations of 1 gram free IAA / liter culture broth are easy to make, but even much higher concentrations are possible, depending on the micro-organism used.

After ending the fermentation, the micro-organism may be lysed and a powder enriched in free IAA may be obtained by spray drying or any other convenient way of drying the culture broth. Other techniques may be used to remove liquids partly or completely.

The term "free IAA" is used herein to indicate that the free IAA is in the free or acid form, whereas the term "conjugated IAA" refers to IAA that is conjugated via ester linkages or via amide linkages.

As long ago as 1956, the effects of free IAA on humans were studied, and it was shown that single doses of 0.1 g/kg body weight were non-toxic (Mirsky A and Diengott D, Hypoglycemic action of indole-3-acetic acid by mouth in patients with diabetes mellitus, *Proc. Soc. Exp. Biol. Med.* 93: 109-110.1956). In 1964, it was found that photo-oxidation products of free IAA acted as growth inhibitors of micro-organisms (Still C, Fukuyama T and Moyed H, Inhibitory Oxidation Products of Indole-3-acetic acid, *J. Biological Chemistry*, 240.6,2612-2618,1964).

Also, the medical use of free IAA and some of its derivatives has previously been described. EP 1.296.676 describes the use of free IAA as a pharmaceutical, in

particular for treating neoplastic disease in humans. WO 02/080906 describes the use of free IAA for treating endometriosis in women. Nachson et al. (Feed and Chemical Toxicology 41, 745-752) reported the effect of some free IAA derivatives (indole-3-carbinol and 3,3'-diindolylmethane) on the proliferation and induction of apoptosis in human prostate cancer cell lines whereas Rossiter et al. (Bioorganic & Medicinal Chemistry Letters, 12, 2523-2526) as well as Folkes et al. (Biochemical Pharmacology 63, 265-272) described the use of free IAA and some derivatives in enzyme-pro-drug directed cancer therapies.

Animal feed comprising IAA has been described in the art, for instance, US-A-2925341 discloses a feed additive which comprises 10-50 mg of indole acetic acid per kilogram of feed.

It was found in our studies that phytohormones and beta-glucans appeared to work in a wide range of concentrations for improving the fertility of animals. The optimal concentrations may vary between different species, however, the skilled person will know how to obtain an optimal concentration for a given species, for instance by titration of the desired compound into the animal feed and testing when this would have the optimal effect. The following may serve as guidance in this process.

A skilled person will appreciate that the amount of free IAA in the ready to use feed has to be adjusted in order to supply the animal with an effective amount of free IAA. In order to adjust the free IAA concentration in the feed so that a certain daily intake of free IAA is achieved, an estimate has to be made of the feed intake of an animal or animal group. A skilled person is aware of the feed intake of a particular (kind or group of) animal(s). Typically, the feed intake per day is between 0.5 and 10% of the body weight of the animal, with occasional exceptions as high as 20 %. Elderly animals tend to eat less and are considered to have a feed intake per day between 0.1 and 5 %, typically of 1% of their body mass.

It was found that fertility of animals was improved when free IAA was provided in their feed in the range of 0.004 and 40 mg per kilogram live weight per day (mg/kglw/day). Optimum between cost and benefit was reached in concentrations between 0.04 and 4 mg/kglw/day, in particular feeding with 0.4 mg/kglw/day free IAA was very effective.

The invention therefore also relates to a process for improving the fertility of an animal wherein the animal is fed with free IAA provided in the feed in the range of 0.004 and 40 mg per kilogram live weight per day (mg/kglw/day).

It was also found that, within the family of beta-glucans, in particular 1,3 and

1,6 beta glucans were very useful to improve the fertility of animals. A particularly good source of such 1,3 and 1,6 beta glucans may be found in preparations of *Agaricus blazei murill* (ABM) or yeast cell walls. Animal feed supplemented with 1 to 1000 mg/kg/day of dried *Agaricus blazei murill* was found to produce the desired effect of improving the fertility of animals. This corresponds to approximately 0.1 to 100 mg/kg/day of 1,3 and 1,6 beta glucans. Excellent results were obtained when the animals were fed with 1 to 10 mg/kg/day of 1,3 and 1,6 beta glucans, optimum of cost benefit was found to be around 5 mg/kg/day, corresponding to 50 mg/kg/day of dried ABM.

The invention therefore also relates to a process for improving the fertility of an animal wherein the animal is fed with 1,3 and 1,6 beta glucans in the range of approximately 0.1 to 100 mg/kg/day.

Animal feed comprising beta-glucans is readily available. For instance, WO 02/091850 A discloses an animal feed which comprises 100-1000mg of beta-glucans per kilogram of feed. Also, WO 02/37988, WO 2004/066863, WO 2004/014320 and US 2005/020490 A1 disclose compositions which comprise beta-glucans, and which may be suitable for use as an animal feed in the present invention. Such animal feed has also been described in Hiss and Sauerwein, *Journal of animal physiology and animal nutrition*, 87, 2003, pp2-11, Deblackwell, Berlin.

The effect of improving the fertility of animals was also observed when the feed of animals was supplemented with Gibberelin or Gibberellic acid. The optimal concentrations here were found to be within the range of 0.0004 and 4 mg/kg/day. The effect of improving the fertility of animals was particularly pronounced in the range of 0.004 and 0.4 mg/kg/day. Optimal results were achieved between 0.01 and 0.1 mg/kg/day, such as 0.04 mg/kg/day.

Suitable feed substances that may be used in the present invention have been described. US-A-2943938 and Svihus et al. (*Journal of Animal Science*, 64, 1997, p257-272) describe an animal feed which may comprise suitable amounts of giberellic acid per kilogram of composition.

As a consequence, the invention is therefore also directed towards the use of any of the substances described above, for the preparation of a medicament for improving the fertility of animals.

EXAMPLES

Example 1 Microbiological production of a preparation containing free IAA

Azospirillum brasilence Sp7 (ATCC) was obtained as an agar culture in a culture tube. LB medium was used to grow the strain overnight at 28 °C at 175 rpm. Glycerol was added to the culture up to 10 %, mixed and divided over Nalgene creovials and frozen at – 80 °C. Stocks were stored at – 80 °C in creovials.

To prepare a seed culture of A. brasilence, one stock (1.2 to 1.8 ml) was thawed and added to 1 liter of LB medium and grown for about 20 h at 28 °C and 175 rpm to an Optical Density (OD620 nm) of about 2.5.

A 10 litre fermentor was rinsed with water and the pH electrode was calibrated. Nine litre of LB medium was prepared and 1 g/l L-Tryptophan and 0.1 g/l free IAA was added. The medium was entered into the fermentor together with 2 ml of anti foam. The fermentor was sterilised for 30 min at 121 °C. After cooling down to 28 °C, the O2 probe is calibrated with N2 and O2, 0 and 100 % air saturation respectively.

The seed culture is transferred to the fermentor via a flask and tubing which are separately sterilised in an autoclave. When the addition is completed the tubing and flask are removed and the fermentation is started with the following parameters:

Stirrer speed	400 rpm
Temperature	28 °C
Aeration	0.75 NI/min
PH	7

After 15 min a sample is taken to measure the OD620 nm and check the pH. Samples are taken at certain intervals to quantify the growth of A. brasilence. When the growth rate declined extra medium was added to ensure that enough biomass was formed for the production of free IAA. It was found that the production of free IAA started when the active growth phase ended and continued for a prolonged period. The course of the free IAA concentration was followed by LC-MS. When the concentration of free IAA was at a level of about 1 g/l, the fermentation was terminated and the cells were harvested and lysed by means of a nonojet homogeniser at about 1400 bar. The remaining supernatant and the lysed cells were sterilised and spray dried to yield the desired product formulation.

Example 2 Preparation of pig feed containing beta glucans

An amount of 50 gram of dried Agaricus Blazei Murill (Agaricus Farm), a natural source of beta-glucans was suspended in 100 ml of olive oil. A pig feed according

to the invention was prepared by vacuum impregnating ten kilogram of the usual commercially available pig feed with 100 ml of the oil suspension. Control feed was prepared by vacuum impregnating the same amount of feed with only olive oil.

Example 3 Preparation of pig feed containing plant growth hormones

An amount of the spray dried formulation as described in example 1 corresponding to 400 milligram of free IAA was suspended in 100 ml of olive oil. A pig feed according to the invention was prepared by vacuum impregnating ten kilogram of the usual commercially available pig feed with 100 ml of the oil suspension. Control feed was prepared by vacuum impregnating the same amount of feed with only olive oil.

Example 4 Preparation of pig feed containing both beta glucans and plant growth hormones

An amount of 50 gram of dried Agaricus Blazei Murill (Agaricus Farm), a natural source of beta-glucans and an amount of the spray dried formulation as described in example 1 corresponding to 400 milligram of free IAA were suspended in 100 ml of olive oil. A pig feed according to the invention was prepared by vacuum impregnating ten kilogram of the usual commercially available pig feed with 100 ml of the oil suspension. Control feed was prepared by vacuum impregnating the same amount of feed with only olive oil.

Example 5 Improving the fertility of sows.

Objective of the study was to determine whether a diet supplemented with free IAA and/or beta-glucans could improve the fertility of sows.

The weaning period of porcine species constitutes a particularly delicate moment from the physiological, metabolic and endocrine point of view. Numerous studies reported that few days before the weaning as well as few days after that, a greater energetic and amino acid food contribution is fundamental in order to adequately stimulate follicular and ovulatory dynamics and therefore to obtain a better reproductive efficiency with favourable implications, mainly on management.

Several sows do not respond to this type of nutritional treatment (higher energy plus amino acid complement). The reproductive efficiency lowers during summer-autumn period; this feature is described as negative seasonal effect.

In order to regulate follicular dynamics and ovulation rate, one of the most frequent protocols is the hormonal treatment by using exogenous gonadotropins. The

hormonal treatments with exogenous substances are currently prohibited by most health guidelines and from a perspective of animal well-being. This holds in particular for the production lines of biological pig.

The experiment was performed in three well-managed animal farms specialised in pork production for the traditional pork butchery (ham, for example). Forty lactating sows were randomly distributed into four groups of 10 sows, three groups were fed with feed according to the invention, one group received control feed. The animals received the feed the 3 last days of lactation until 3 days after weaning.

Inclusion criteria for the study were:

- Sows that delivered from at least 21 days
- Sows not submitted to veterinary treatments
- Sows without post-partum anorexia
- Sows with prolificacy of 10 or more at the last delivery
- Sows that nurses at least 9 piglets

The BCS is not comprised in the inclusion criteria; backfat will not be considered.

Exclusion criteria were:

- Sows that the breeding technician will eliminate after the weaning
- Sows subordinates to veterinary treatments
- Sows with viral infections, in particular PRSV
- Sows with syndrome mastitis-metrite-agalaxia
- Sows with limb lesions
- Sows with fever and anorexia during lactation

The administration of the feed supplement was performed in the morning in concentrations indicated in table 1. After weaning, the pigs were placed in “fecundation” cages and stimulated two times per day with boars skilled for oestrous detection. Oestrous sows were then inseminated by a technician following the classical protocol.

Sows were diagnosed for pregnancy in the 3rd or 4th week after insemination using ultrasonography. The prolificacy of each sow was registered after parturition.

From the first results of the experiment it was observed that sows treated with the feed according to the invention had a better metabolic and endocrine status and presented an optimal weaning/oestrus period (5 days) as well as a better follicular dynamics which all translated into a higher percentage of pregnant sows and a higher number of offspring.

Table 1

	feed additives for pig feed
Group 1	0.4 mg/kg/w/day free IAA
Group 2	50 mg/kg/w/day of dried ABM corresponding to 5 mg/kg/w/day of 1,3 – 1,6 beta glucan
Group 3	0.4 mg/kg/w/day free IAA plus 50 mg/kg/w/day of dried ABM corresponding to 5 mg/kg/w/day of 1,3 – 1,6 beta glucan
Group 4 (control)	none

CLAIMS

1. Use of an animal feed comprising between 0.05 and 500 milligram of beta-glucans per kilogram of feed and/or between 0.1 and 1000 milligram of phytohormones per kilogram of feed for improving the fertility of animals.
2. Use according to claim 1 wherein the phytohormone is selected from the group consisting of indole-3-acetic acid, cytokinin, gibberellin, gibberellic acid, ethylene, abscisic acid, brassinosteroids, jasmonic acid and salicylic acid.
3. Use according to claims 1 or 2 wherein the beta glucans mainly consist of 1,3 and/or 1,6 glucans.
4. Use according to claims 1 - 3 comprising between 0.5 and 50 milligrams, preferably between 1 and 10 milligrams of beta-glucans per kilogram of feed.
5. Use according to claims 1 - 4 comprising between 0.1 and 1000 milligram, preferably between 1 and 100 milligrams, even more preferably between 10 and 100 milligrams of phytohormones per kilogram of feed.
6. Use according to claims 1 – 5 wherein the phytohormone is free IAA.
7. Use according to claims 1 - 5 wherein the phytohormone is gibberellic acid.
8. Use according to any of claims 1 - 7 for the preparation of a medicament for improving the fertility of animals.
9. Use according to any of claims 1 – 8 wherein the animal is female.
10. Animal feed comprising between 0.05 and 500 milligram of beta-glucans per kilogram of feed and between 0.1 and 1000 milligram of phytohormones per kilogram of feed.

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2006/061673

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>TONIOLLI, R., BUSSIÈRE, J., COUROT, M., COMBARNOUS, Y.: "Effect of indole-3-acetic acid (plant auxin) on boar sperm motility and pregnancy and proliferacy rates after freezing and thawing"</p> <p>REPRODUCTION IN DOMESTIC ANIMALS, vol. 33, 1998, pages 33-38, XP008052941</p> <p>DEBLACKWELL WISS. VERLAG, BERLIN</p> <p>the whole document</p>	1-10
X	<p>BOMFORD, M.: "Food and reproduction of wild house mice III. Experiments on the breeding performance of caged house mice fed rice-based diets"</p> <p>AUSTRALIAN WILDLIFE RESEARCH, vol. 14, 1987, pages 207-218, XP008052928</p> <p>the whole document</p>	1-10
X	<p>EL MOFTY, M. M.: "Induction of sexual reproduction in opalina sudafricana by injecting its host bufo regularis with gibberellic acid"</p> <p>INTERNATIONAL JOURNAL OF PARASITOLOGY, vol. 4, 1974, pages 203-206, XP002346412</p> <p>GBPERGAMON PRESS</p> <p>the whole document</p>	1-10
X	<p>XIAO, Z., TRINCADO, C. A., MURTAUGH, M. P.: "beta-Glucan enhancement of T cell response in swine"</p> <p>VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY, vol. 102, 2004, pages 315-320, XP002346411</p> <p>NLAMSTERDAM</p> <p>the whole document</p>	1-10

INTERNATIONAL SEARCH REPORT

International application No.
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Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: —
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-9 is directed to a method of treatment of the animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the composition.
2. Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2006/061673

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
RU 2063749	C1	20-07-1996	NONE