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(54) Titre : PROCÉDE POUR AMÉLIORER UNE RESORPTION DE MINÉRAL CHEZ DES POISSONS ET CRUSTACÉS D'ÉLEVAGE  
(54) Title: METHOD FOR IMPROVING MINERAL RESORPTION IN FARMED FISH AND CRUSTACEAN

(57) **Abrégé/Abstract:**

One uses advantageously, as feed, feed component or feed additive a composition comprising an efficient amount of vitamin D metabolites or any source of same for improving or enhancing resorption of minerals, especially phosphor and calcium in farmed fishes and crustaceans. One further provides mean enabling a substantial reduction of phosphor pollution in aquaculture when feeding fishes and crustaceans by means of a dedicated feed having reduced phosphor content and supplemented in the same time with such a feed, feed component or feed additive.

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(54) Title: METHOD FOR IMPROVING MINERAL RESORPTION IN FARMED FISH AND CRUSTACEAN

(57) Abstract: One uses advantageously, as feed, feed component or feed additive a composition comprising an efficient amount of vitamin D metabolites or any source of same for improving or enhancing resorption of minerals, especially phosphor and calcium in farmed fishes and crustaceans. One further provides mean enabling a substantial reduction of phosphor pollution in aquaculture when feeding fishes and crustaceans by means of a dedicated feed having reduced phosphor content and supplemented in the same time with such a feed, feed component or feed additive.



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## Method for improving mineral resorption in farmed fish and crustacean

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### Field of the Invention

The invention relates generally to the field of fish and crustacean farming, in particular  
10 to specific feed components or additives suitable to feed fishes or crustaceans. More  
specifically the invention relates to means useful for improving mineral resorption in fishes  
and crustaceans.

### 15 Background of the Invention

Supplying the growing world population with food is highly challenging. One of the  
most valuable nutritive components is protein which comes typically from meat of land  
animals but also from fish. Consumption of fish and crustacean is a meat source since  
20 prehistoric times. However, in the last decades, ocean fishing came to a critical point in which  
over-fishing reduced catches of many marine species such as sardines, tuna and herring. Also,  
modern fishery practices destroy the ocean floor and this impairs hatching ground for many  
marine animals.

25 Fish farming is therefore a suitable alternative to ocean fishing. So, for example salmons have  
the best feed conversion ratio (FCR) of all farmed animals

However, fish and crustacean aquaculture has also its shortcomings, which needs to be  
overcome in order to make aquaculture sustainable:

30

1. Stocking density as a cause of stress and growth anomalies
2. Over fertilization of coastal areas caused by excreta and wasted feed
3. The use of fish meal and fish oil as feed components

Such draw-backs are recognized by the industry and intensive research is ongoing to improve the situation and make aquaculture more sustainable. One of the proposed strategies consists to replace the use of fish meal and oil by raw material of plant origin and to correct eventual imbalances. Another strategy is to find additives which would induce a better use of other  
5 nutrients.

In addition, fishes and crustaceans in aquaculture do not have access to the natural feed components in marine or freshwater environment like essential nutrients e. g. vitamins from plankton. On the other hand freshwater and seawater contain some minerals in sufficient  
10 amounts but are deficient in others like e.g. phosphorus.

In contrast to calcium phosphorus is a growth-limiting nutrient for aquatic animals and must be supplemented by feed. Phosphorus is together with calcium a main component of bone and scales in fish, but also in the exoskeleton of crustaceans. In addition, phosphorus is an  
15 essential element in muscle function.

It is therefore of high interest to find methods which help to make an efficient use of such nutrients and not to waste it in the environment.

20 The invention obviates adequately all the obstacles still met in fish and crustacean farming and responds adequately to an obvious unmet need in aquaculture.

The present invention relates, in particular, to a method which improves calcium and phosphorus uptake in teleost fishes and crustaceans and that improves growth and animal  
25 body composition and that yields better meat quality. This allows an easy and profitable reduction in such components in the feed whereas it still allows achieving the desired performance.

The invention is defined in the claims appearing here below.

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## Summary of the Invention

A first object of the invention is a composition for use in improving or enhancing resorption of minerals in fishes and crustaceans which comprises as an active ingredient  
5 Vitamin D metabolites or any source of same.

Another object of the invention is a feed or a feed material useful for improving or enhancing resorption of minerals in fishes and crustaceans which comprises as active ingredient Vitamin D metabolites or any source of same in combination with a source of  
10 carbohydrates, a source of lipids and a source of proteins.

Still another object of the invention is a feed additive useful for improving or enhancing resorption of minerals in fishes and crustaceans which comprises as an active ingredient Vitamin D metabolites or any source of same.  
15

Still another object of the invention is a method for reducing phosphorus pollution in aquaculture, especially in fish and crustacean farming which comprises feeding said fishes and crustaceans by means of a feed or a feed material mentioned here above.

20 Additional objects of the invention shall appear in the specification below.

## Detailed description of the Invention

25 According to the invention the terms "Vitamin D metabolites" mean either 25-Hydroxyvitamin D3 (Calcidiol, CAS number: 19356-17-3) , 25-Hydroxyvitamin D2; 1,25-Dihydroxyvitamin D3 (Calcitriol, CAS 32222-06-3) , 1,25-Dihydroxyvitamin D2; 1- $\alpha$ -Hydroxy-cholecalciferol , 1- $\alpha$ -Hydroxyergosterol or 1,25-Dihydroxyvitamin D3-glycosides whereas the term "glycosides" defines glucoside, fructoside, galactoside units or any hexose  
30 and pentose unit bound to the aglycon. 1,25-Dihydroxyvitamin D3-glycosides represent one of the preferred Vitamin D metabolites used within the frame of the invention.

According to the invention the terms "source of Vitamin D metabolites" comprise preparations or part of plants, and namely leaves, obtained from plants like *Solanum*

*glaucophyllum*, *Cestrum diurnum*, *Trisetum flavescens* and *Nierembergia veitchii*. That list, however, is not limitative.

According to the invention said vitamin D metabolites can be applied alone, i.e. as unique  
5 vitamin entity or in addition to regular vitamin D whereby the addition to regular Vitamin D3  
represents the preferred application.

According to the invention the term “fish” comprise any species suitable to farming or  
aquaculture and in particular marine fishes such as Atlantic salmon, milkfish, halibut, Atlantic  
10 cod, Pacific salmon, turbot, sole, sea bass, finfish and eels as well as freshwater fishes like  
trout e.g. rainbow trout, carps, tilapia, catfish, pangasius and bream.

According to the invention the term “crustaceans” comprise any species suitable to farming or  
aquaculture, ant in particular marine shrimps and prawns like e.g. tiger prawns and fresh  
15 water prawns, crabs and crayfish.

According to the invention the term “mineral” includes phosphorus, calcium and magnesium;  
phosphorus is first of all of concern within the frame of this invention as being a major source  
of pollution in fish farming.  
20

According to the invention the terms “mineral resorption” means intestinal resorption or  
resorption through the scales (fishes) or through the shell (crustaceans).

According to the invention *Solanum glaucophyllum* plant material represents a preferred  
25 source of Vitamin D3 metabolites, especially of 1,25-dihydroxyvitamin D3-glycosides. This  
plant material is provided either from wild or from cultivated plants or hybrids of same like  
e.g. “HERVIT®” according to Plant Variety Certificate EU 25473.

As plant material one can use either dried leaves of said plant or any water extract of same,  
30 any water/alcohol extract of same or any enriched and purified water/alcohol extract of same  
(see examples below).

According to the invention 1,25-dihydroxyvitamin D3-glycosides are provided to the fish and  
crustacean in their feed at a daily dose of about 0.1 µg to about 100 µg of 1,25-

dihydroxyvitamin D3-glycosides per kg fish feed or crustacean feed, a dose which is usually expressed analytically as  $\mu\text{g}$  of 1,25-dihydroxyvitamin D3.

5 One uses preferably from about 0.2 to about 20  $\mu\text{g}$  1,25-dihydroxyvitamin D3-glycosides per kg of feed material and still more preferably from about 0.5 to about 10  $\mu\text{g}$  1,25-dihydroxyvitamin D3-glycosides per kg of feed material. The vitamin D metabolites can be incorporated to any feed material or component dedicated to any state of development of fish and crustacean.

10 Such feed or feed material can further comprise from about 25 $\mu\text{g}$  to about 75  $\mu\text{g}$  regular (synthetic) Vitamin D, e.g. (synthetic) Vitamin D3 per kg feed or feed material.

When applying 1,25-dihydroxyvitamin D3-glycosides in accordance with the provisions here above, and in particular when performing the application of a single dose of 1,25-dihydroxyvitamin D3-glycosides - e.g. provided as water soluble extract of *Solanum glaucophyllum* according to WO2009/129818) - or of synthetic 1,25-dihydroxyvitamin D3, 25-hydroxyvitamin D3 or of 1- $\alpha$ -hydroxyvitamin D3 to adult Atlantic salmon one observes increased blood levels of 1,25-dihydroxyvitamin D3. When comparing the above mentioned fishes to untreated control subjects one also observes a substantial increased uptake of calcium and phosphorus from the standard feed provided to the said fishes.

20 According to another series of test performed with juvenile Atlantic salmon and making use of 1,25-dihydroxyvitamin D3-glycosides in the form of a water soluble extract of *Solanum glaucophyllum* (see above) one observes, after 30 days of feeding, a definite better growth rate, a better feed conversion and lower bone and scales deformations or distortions. Similar results have been achieved when using Panbonis® 10 as an alternative source of 1,25-dihydroxyvitamin D3-glycosides.

30 According to another object the invention one can decrease advantageously the phosphorus content in the feed or the feed component used in aquaculture when reducing the initial standard and generally recommended proportion for a given species and stage of development by 10 to 50 % of said initial proportion while adding thereto an efficient amount of Vitamin D3 metabolites referred to here above and while still achieving the same end performance, i.e. growth rate, meat and bone quality.

For example feeding adult Atlantic salmon with different doses of 1,25-dihydroxyvitamin D3-glycosides – provided as an extract of *Solanum glaucophyllum* (see above) - to diets with graded reductions of added feed phosphorus over 60 days resulted in a better growth rate, a better feed conversion and less phosphorus in excreta when compared to a non-phosphorus-reduced control group. Similar results have been achieved by using a combination of Panbonis® 10 together with a feed having reduced phosphorus content as compared to a control group where phosphorus content of their diet was not reduced.

Thus, by means of the invention, one can advantageously reduce the phosphorus pollution in aquaculture, especially in fish and crustacean farming when feeding said fishes and crustaceans by means of a feed or feed additive mentioned here above.

When feeding adult Atlantic salmon with different doses of 1,25-dihydroxyvitamin D3-glycosides– provided as an extract of *Solanum glaucophyllum* (see above) - to diets with graded reduction of added fish meal and fish oil and by replacing same with plant proteins and plant oils one observes, nevertheless, the same growth performance than that of the non-reduced control group.

Similar results have been achieved when using Panbonis® 10 as an alternative source of 1,25-dihydroxyvitamin D3-glycosides to a feed material in which a fraction of fish meal and of fish oil was replaced by plant proteins and vegetal oils, when compared to non-replaced control feed material.

When fishes or crustaceans are fed according to the invention one observes a definite improvement of their well-being which is reflected, in particular, in the quality of their meat and their bone and scales health and, also, in their growth rate and their feed conversion ratio.

One could also improve substantially the growth rate and reduce at the same time the bone deformation in farmed fishes and crustaceans by means of a feed or feed material or feed additive as referred to here above.

Consequently the invention further provides a method for improving growth rate and well-being of farmed fishes and crustaceans when a part of fish meal and fish oil is replaced by

vegetable proteins and oils by means of a feed or feed additive referred to here above. Following such a way one is today able to correct efficiently potential imbalances which would be caused by these feed substitutions.

- 5 The invention still further provides a method for improving growth rate and well-being of farmed fishes and crustaceans when a part of fish meal and fish oil is replaced by vegetable proteins and vegetal oils and supplemented at the same time with a feed or feed additive mentioned here above and in combination with the use of phytase enzymes when anti-nutrient phytic acid is present in said vegetable oils and proteins.

10

The incorporation of selected Vitamin D3 metabolites, especially of 1,25-dihydroxyvitamin D3 glycosides, is performed according to the techniques usually applied in this field for producing pellets or crumbles or similar feed material, namely extrusion, coating, spraying, etc. The selected metabolites are primarily mixed with the carbohydrates, a first portion of lipids and the proteins and then subjected to extrusion to afford granule or pellet cores of. A protective coating made of the second portion of selected lipids is then sprayed onto the cores to provide the ready to use pellets.

15

## 20 **Examples**

### **Preparation of a Solanum glaucophyllum plant extract**

A selected batch of dry leaves of Solanum glaucophyllum has been macerated under agitation at 40 - 60 °C for about 24 hours with the 5 to 12 liter per kg of a water/ethanol mixture.

25

Previous analytical assessment of the content in 1,25-dihydroxyvitamin D3-glycosides of various batches of dry leaves material available in the storehouse led to prepare an 85/15 in volume water / ethanol mixture. The use of the proper mixture allows the achievement of the desired content of vitamin D3 derivative – approx. 100 ppm in this example. The liquid fraction was separated and set aside whereas maceration was repeated for a second turn of 24 hours.

30

The collected water / ethanol extracts were then combined and added with ascorbic acid in a ratio of 0.25 % by weight and pH of same was adjusted to 5 – 6.5 by means of a food-compatible acid, citric acid in this particular case.

- 5 The stabilized extract was then filtered and concentrated to approx. 30 to 50 % of dry matter content by means of vacuum drying technique and the resulting concentrated was poured onto a cationic exchanger resin equilibrated in its H<sup>+</sup> form.

- The collected effluent was filtered and then subjected to UHT sterilization (125° C for 1 min).  
10 After analytically assessment of the 1,25-dihydroxyvitamin D<sub>3</sub>-glycosides content, a specific amount of excipient, preferable maltodextrin, lactose or corn starch, is added the sterilized material in order to standardize the final content of active ingredients in the plant extract – 100 ppm of 1,25-dihydroxyvitamin D<sub>3</sub>-glycosides.

- 15 The standardized mixture is eventually spray dried or vacuum dried in a conventional spray drier to afford the desired *Solanum glaucophyllum* plant extract in powder form.

- Alternatively *Solanum glaucophyllum* plant material can be subjected to an extraction by percolation with the water / ethanol solvent mix referred to above followed by the addition of  
20 stabilizing additives like e.g. ascorbic acid and pH adjustment, before subsequent UHT treatment and drying (vacuum drying or spray drying).

#### **Preparation of a feed component (or feed additive): Panbonis® 10**

- 25 Wheat middling (excipient) is intimately milled with dried leaves of *Solanum glaucophyllum* and then processed until obtaining a homogeneous powdered material. The inactive excipient is added to the dried leaves in order to obtain a standardized product comprising 0.001 % (weight) of 1,25-dihydroxyvitamin D<sub>3</sub>-glycosides (measured analytically as “active Vitamin  
30 D<sub>3</sub>” i.e. as 1,25-dihydroxyvitamin D<sub>3</sub> or, expressed differently, as 1,25(OH)<sub>2</sub> D<sub>3</sub>); Panbonis® 10 is more conveniently characterized here below as comprising 10 µg 1,25-dihydroxyvitamin D<sub>3</sub> / kg.

The analytical content of the excipient may vary according to the following range: 8 – 12 % (weight) of crude fibers, 14-25 % (weight) of starch. The mill processing is set in order to afford a particle size distribution comprised between ca. 50 and ca. 1000  $\mu\text{m}$ .

5

## Fish feeding experiments

### Experiment no 1

To a total of 116 brown trout (*Salmo Salar*) having each a body weight extending from 250 g to 500 g to which was periodically provided a commercial grower feed (crude protein: 46%; crude fat 12%; carbohydrate 20%; crude fibers 3%; ash 9%; calcium “.0%; P 1.5%; vitamin A 5000IU/kg; vitamin D3 2500 IU/kg) and moving in separate tanks, one gave to each of same one capsule containing 1.75  $\mu\text{g/kg}$  bodyweight of 1,25-Dihydroxyvitamin D3. At different times the fishes were anesthetized and blood was withdrawn from them, serum samples were prepared and submitted to analytical quantification of 1,25-Dihydroxyvitamin D3, calcium and phosphorus.

15

**Table 1: Serum 1,25(OH)2D3, calcium and phosphor values**

Blood parameter	Time 0 hrs.	Time 12 hrs.	Time 24 hrs.	Time 48 hrs.	Time 72 hrs.
1,25(OH)2D3 [pg/ml]	51	246	1254	391	72
Ca [mmol/L]	3.06	2.53	2.41	2.38	2.59
P [mmol/L]	3.18	3.18	3.70	4.24	4.48

20

One observes a blood serum increase of 1,25(OH)2 D3 with a peak concentration at 24 hours followed by a progressive decrease down to the pretreatment level. A slight increase in calcium and phosphorus levels can only be seen 48 hours after administration.

25

## Experiment no 2

Thirty-five juvenile rainbow trout (*Oncorhynchus mykiss*) having an average body weight of 19 gram were kept per tank in 4 replicates per treatment. The duration of the feeding experiment extended over 73 days at a water temperature of 14°C. Fishes were fed once per day to apparent satiation with a diet according to Table 2. Panbonis® 10 was used as the source of 1,25(OH)<sub>2</sub> D<sub>3</sub>, in a ratio of 200 mg/kg diet, respectively of 1000 mg/kg diet.

**Table 2: Composition of the diet of Experiment 2**

10

Raw materials (%)	Control	Panbonis-1	Panbonis-2
Fish meal	10.0	10.0	10.0
Wheat flour	12.0	12.0	12.0
Vegetable protein	67.8	67.8	67.8
Fish meal	10.0	10.0	10.0
Vitamin premix	0.4	0.4	0.4
Mineral premix	6.0	6.0	6.0
Cellulose powder	1.0	1.0	0.9
Panbonis® 10	----	0.020	0.100
Fish oil	3.0	3.0	3.0
Total	100	100	100

**Table 3: Result providing by the parameters applied in Experiment 2**

Indices	Control	Panbonis-1	Panbonis-2
Fish meal (%)	10	10	10
Initial fish number	35	35	35
Starting biomass (g)	674	668	666
Average body weight (g)	19.2 ± 1.5	19.1 ± 1.4	19.0 ± 1.3
Final biomass (g)	2,036	2,134	2,179
Average body weight (g)	58.2±12.9	61.0±13.5	62.2±15.6
Survival rate (%)	100	100	100
Weight gain (g)	1,362	1,466	1,512

Average weight gain (%)	302	319	327
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Serum analysis	Control	Panbonis-1	Panbonis-2
Panbonis® 10 (%)	----	0.020	0.100
1,25(OH) <sub>2</sub> D <sub>3</sub> (µg/ml)*	89.2±15.0	102.3±2.8	133.1±31.2
* Serum samples collected from 3 fishes were mixed to one specimen for the detection of 1,25(OH) <sub>2</sub> D <sub>3</sub> .			

On observes a dose-dependent increase in average weight gain as result of the addition of the 1,25-Dihydroxyvitamin D<sub>3</sub>-glycosides provided by the various dosages of Panbonis® 10 The increase of serum concentration in 1,25-Dihydroxyvitamin D<sub>3</sub>-glycosides - measured as 5 1,25(OH)<sub>2</sub> D<sub>3</sub> - was also dose-dependent. The survival rate of the fishes was 100%.

### Experiment no 3

10 Thirty-five juvenile rainbow trout (*Oncorhynchus mykiss*) of an average body weight of 28 gram were spread over 6 tanks during the whole treatment. Duration of the feeding experiment was 32 days at a water temperature of 14°C. Fish were fed once per day to apparent satiation with a diet as shown in Table 4. Panbonis was used as test article in a concentration of 0.1, 0.2, 1.0, 5.0 and 10 g/kg diet.

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**Table 4: Composition of the diet of Experiment 3**

Raw Materials (%)	Control	Panbonis 0.1g/kg	Panbonis 0.2g/kg	Panbonis 1.0g/kg	Panbonis 5.0g/kg	Panbonis 10g/kg
Wheat flour	16.64	16.64	16.64	16.64	16.64	16.64
Vegetable protein	44	44	44	44	44	44
Fish meal	25	25	25	25	25	25
Vitamin premix	0.72	0.72	0.72	0.72	0.72	0.72
Mineral premix	1.2	1.2	1.2	1.2	1.2	1.2
Others	4.94	4.94	4.94	4.94	4.94	4.94
Defatted rice bran	1	0.99	0.98	0.9	0.5	0

Panbonis® 10	0	0.01	0.02	0.1	0.5	1
Fish oil	6.5	6.5	6.5	6.5	6.5	6.5
Total	100	100	100	100	100	100

**Table 5: Results of Experiment 3**

Indicators	Control	Panbonis 0.1g/kg	Panbonis 0.2g/kg	Panbonis 1.0g/kg	Panbonis 5.0g/kg	Panbonis 10g/kg
Fish meal(%)	25	25	25	25	25	25
Start biomass (g)	663	658	663	662	662	663
Aver. body weight (g)	28.8 ± 2.6	28.6 ± 2.3	28.8 ± 2.8	28.8 ± 2.2	28.8 ± 2.6	28.8 ± 2.6
Final Biomass (g)	1,260	1,248	1,304	1,494	1,410	1,301
Aver. body weight 0(g)	54.8 ± 6.8 <sup>a</sup>	54.3 ± 7.6 <sup>a</sup>	56.7 ± 5.6 <sup>a</sup>	65.0 ± 7.0 <sup>b</sup>	61.3 ± 5.5 <sup>b</sup>	56.6 ± 6.0 <sup>a</sup>
Survival rate (%)	100	100	100	100	100	100
Weight gain (g)	597	590	642	832	749	638
Aver. weight gain (%)	190	190	197	226	213	198
Ca content (g/fish)	0.42	0.39	0.43	0.54	0.46	0.44
P content (g/fish)	0.42	0.4	0.42	0.56	0.49	0.43

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As result of this short term experiment it one observes a similar outcome than that of Experiment 2 in terms of growth performance. The trial shows an increased response for calcium & phosphor deposition in the fish.

10 The optimal dose was found at 1 gram Panbonis® 10 per kg of diet, what corresponds to 10 µg 1,25-Dihydroxyvitamin D3-glycosides per kg of fish diet.

#### **Experiment no 4 (fish meal reduction experiment)**

15

Forty juvenile rainbow trout (*Oncorhynchus mykiss*) having an average body weight of 26 grams in average were kept per tank in 6 replicates per treatment. The feeding experiment

took place over 62 days at a water temperature of 14°C. Fishes were fed once per day to apparent satiation with a diet as shown in Table 6. Panbonis® 10 was used as test article in a concentration of 1.0 g/kg diet.

5 **Table 6: Composition of the diet of Experiment 4**

Raw materials (%)	Control	Control low fish meal	Panbonis 1.0 g/kg
Fish meal	40	25	25
Wheat flour	18.99	14.64	14.64
Vegetable protein	35	44	44
Fish meal	40	25	25
Vitamin premix	0.35	0.72	0.72
Mineral premix	0.8	1.2	1.2
Fish oil	6.5	8.5	8.5
Others	0.36	4.8	3.3
Panbonis® 10	----	----	0.10
Deffated rice bran	----	1.14	2.54
Total	100	100	100

**Table 7: Results of Experiment 4**

Indicators	Fish meal control	Low fish meal	Panbonis 1.0 g/kg
Initial biomass (g)	1,048	1,034	1,033
Average body weight (g)	26.2±1.9	25.9±2.4	25.8±2.0
Final biomass (g)	3,247	3,058	3,444
Average body weight (g)	81.2±17.0 <sup>a</sup>	76.4±19.5 <sup>b</sup>	86.1±17.1 <sup>a</sup>
Survival rate (%)	100	100	100
Weight gain (g)	2,199	2,023	2,411
Average weight gain (%)	310	296	333

One observes that a reduction in fishmeal affected the weight performance in rainbow trout. However, the addition of 1 g/kg Panbonis® 10 to the diet having a lower content of fish meal improved the growth performance significantly although fishmeal inclusion was reduced in the feed.

5

As a conclusion from experiments 2, 3 and 4 shows that:

- Calcium and Phosphorus utilization can be improved
- Fishmeal replacement is possible
- 10 - Improved growth performance is real
- Improved serum levels of active vitamin D3 are attained.

A further experiment was then performed in which the initial proportion of added phosphor in the diet was reduced.

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#### Experiment 5 (Phosphorus reduction)

20 Thirty juvenile rainbow trout (*Oncorhynchus mykiss*) of 126 grams in average were kept per tank in 4, respective 3 replicates per treatment. Duration of the feeding experiment was 63 days at a water temperature of 15°C in a recirculating system.

Fish feed to feeding table to ensure no feed wastage and optimal feed conversion ratio (FCR) and fish were fed once per day to apparent satiation with a diet as shown in Table 8. Lighting was a constant 24h light regime. Dissolved oxygen was maintained above 7mg/l.

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Positive control diet used commercially used standard P level of 0.90%, whereas the test diets used a P level of 0.70% at 26% below the positive control. Phytase was used at 750FTU/g. Panbonis was included at 0.15 and 0.3 g/kg diet and in conjunction with Phytase.

30 **Table 8: Composition of the basal diet (negative control - NC)**

Ingredient	g/kg diet
Fish meal	220

Soy protein concentrate	210
Wheat gluten	86
Corn gluten	60
Wheat meal	170
Lysine	6
Vitamin-mineral premix	20
Phosphate (MCP)	-
Rapeseed oil	160
Fish oil	68

**Table 9: Composition of the supplemented diet (negative control – NC)**

Treatment code	Panbonis mg/kg	Phytase, U/kg	Inorganic P g/kg	Replicates
Negative control (NC)	-	-	-	4
NC + Phosphate	-	-	2	4
NC + Panbonis® 10	150	-	-	3
NC + Panbonis® 10	300	-	-	3
NC + Phytase	-	750	-	3
NC + Phytase + Panbonis®	150	750	-	3

**5 Table 10: Fish weight at end of experiment, (FCR) & bone ash in Experiment 5**

Parameters	Positive Control	Negative Control	NC + Phytase	NC + Phytase +Pan150	NC + Pan150	NC + Pan300
Fish end weight [g]	454	448	446	448	440	450
FCR	0.87	0.88	0.89	0.88	0.90	0.87
Total bone ash mean	49.8	45.3	50.1	50.3	47.6	45.7

As result, the addition of the 1,25-Dihydroxyvitamin D3-glycosides containing product Panbonis to a diet reduced in phosphorus (from normal 0.9% to 0.7%) resulted in a definite

improvement of the feed conversion ratio and bone ash when the phosphorus reduced diet was supplemented with either Phytase or Panbonis® 10 or a mixture of both.

### Experiment no 6

- 5 15 Japanese prawn (*Marsupenaeus japonica* – average body weight around 6.5 gram in 200L polycarbonate resin tank with (4 tanks ?) - water temperature 14°C - feeding once per day in the morning up to apparent satiation - dried *Solanum glaucophyllum* leaves constitute the source of 1,25-dihydroxyvitamin D<sub>3</sub>-glycosides.

10 **Table 11: Composition of the feed formulation in Experiment 6**

Table 11	Control-5	Test-9	Test-10	Test-11
Panbonis® 10 (%)	-	0.001	0.01	0.10
1,25(OH) <sub>2</sub> Vit D <sub>3</sub> (µg/kg)	-	0.1	1	10
Animal protein	71.00			
Fish meal	1.00			
Mono Calcium Phosphate	4.00			
Vitamin premix	2.00			
Others	22.00	22.00	21.00	21.90
Total	100.00	100.00	1100.00	100.00
Level of Vit D <sub>3</sub> derived from Vitamin premix and fish oil (µg/kg)	188			

**Tables 12, 13 & 14: Results of Experiment 6**

Table 12	Control-5	Test-9	Test-10	Test-11
Panbonis® 10 (%)	----	0.001	0.01	0.10
1,25(OH) <sub>2</sub> Vit D <sub>3</sub> (µg/kg)	----	0.1	1	10
Initial number	45	45	45	45
Average body weight (g)	6.4±0.9	6.5±0.9	6.4±0.8	6.5±0.8

Final number	44	45	44	45
Average body weight (g)	11.6±1.6	11.5±1.3	11.3±1.1	11.7±1.5
Total weight gain (g)	222.0	222.5	210.1	233.2
Average weight gain (%)	181	177	177	180

## Shell hardness \*

Table 13	Control-5	Test-9	Test-10	Test-11
Panbonis® 10 (%)	----	0.001	0.01	0.10
1,25(OH) <sub>2</sub> Vit D <sub>3</sub> (g)	----	0.1	1	10
Shell hardness (g)	175±39.8 <sup>a</sup>	283±118 <sup>b</sup>	315±65.1 <sup>b</sup>	267±55.4 <sup>b</sup>
1st abdominal segment (g)	116.6±31.5 <sup>a</sup>	178.6±26.3 <sup>b</sup>	219.0±51.8 <sup>c</sup>	178.2±49.9 <sup>b</sup>

5 \* Detected by Rheometer CR300 (Sun Scientific Co. Ltd.).

## Calcium content \*

Table 14	Control-5	Test-9	Test-10	Test-11
Panbonis® 10 (%)	----	0.001	0.01	0.10
1,25(OH) <sub>2</sub> Vit D <sub>3</sub> (g)	----	0.1	1	10
Ca content (% in dry matter)	4.42	5.13	5.68	5.19

10 \* 6 shrimps were collected from each treatment and mixed to afford one sample for Ca content measurement.

One observes – see Table 13 – a significant improvement of shell concerning the crustaceans fed with the composition according to the invention, hardness as compared to the control population. Table 14 further shows a parallel improvement of the calcium absorption.

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**WE CLAIM:**

1. Use of a feed for farmed fishes and crustaceans having a reduced graded content of fish meal and fish oil, supplemented with vegetable proteins and vegetal oils supplemented with an efficient amount of 1,25-dihydroxyvitamin D3-glycosides provided from a source of *Solanum glaucophyllum*, CHARACTERIZED in that it is for preparing a food useful in improving growth rate of farmed fishes and crustaceans.
2. Use according to claim 1, CHARACTERIZED in that the vegetable proteins and vegetal oils are further supplemented by means of phytase enzymes when anti-nutrient phytic acid is present in said vegetable oils and proteins.
3. Use according to any of claims 1 or 2, CHARACTERIZED in that the fishes are selected from the group consisting of marine fishes, Atlantic salmon, milkfish, halibut, Atlantic cod, Pacific salmon, turbot, sole, sea bass, finfish, eels, freshwater fishes, trout, rainbow trout, carps, tilapia, catfish, pangasius, and bream.
4. Use according to any one of claims 1 to 2, CHARACTERIZED in that the crustaceans are selected from the group consisting of marine shrimps, prawns, tiger prawns, fresh water prawns, crabs, and crayfish.
5. Use according to any one of claims 1 to 4, CHARACTERIZED in that said 1,25-dihydroxyvitamin D3-glycosides are provided to the fishes or crustaceans in their feed at a daily dose of about 0.1 µg to about 100 µg of 1,25-dihydroxyvitamin D3-glycosides per kg fish or crustacean feed.

6. Use according to any one of claims 1 to 5, CHARACTERIZED in that said 1,25-dihydroxyvitamin D3-glycosides are provided in addition to regular Vitamin D3 originating from other source.