Article

A Feed Additive Containing Encapsulated 6-Phytase within Recombinant Yarrowia lipolytica Cells Produced by Cultivation on Fat-Containing Waste

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Abstract: Feed phytases are purchased as a dry culture medium of secreting producers, mostly micellar fungi. These preparations are required to withstand heating up to 75–80 °C because they are intended for mixing with feed components with subsequent granulation by spray drying. For this reason, many phytases that have a high specific activity at 37 °C and correspond to the optimal pH of intestinal chyme are not used in practice. A novel expression system allowing accumulation of the phytase from Obesumbacterium proteus within yeast Yarrowia lipolytica was proposed. Encapsulation increases thermal stability of the enzyme from 55 °C up to 70 °C. The obtained preparation exhibited a high impact on the daily weight gain of a weaned mouse model fed a phosphorus-deficient diet at a dosage 165 phytase activity units (FYT)/kg, whereas a commercial phytase preparation—Ladozyme Proxi derived from Aspergillus ficuum—did not improve the daily weight gain even at the dosage of 15,000 FYT/kg.

Keywords: phytase; microencapsulation; daily weight gain; weaning mice; Obesumbacterium proteus; Yarrowia lipolytica

1. Introduction

Phytase is a key enzyme used in feed production [1]. This is explained by the fact that phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate [IP6]) is the principal storage form of phosphorus and a variety of other vital chemical elements in plants, representing approximately 75–80% of the total phosphorus in plant seeds [2]. A large portion of phosphorus in cereals and oilseeds is bound as phytate, which cannot be assimilated due to a low endogenous phytase level in the animal intestine [3]. The available phosphorus (aP) requirement of animal diets was estimated to be 0.35–0.45% [4]. A diet composed of ~65% corn meal and ~30% soybean meal contains 0.13% aP [5]. Recalculation of these data shows that the complete release of the inorganic phosphate from the phytate contained in this type of the diet can adjust the aP level up to 0.48%.

A broad range of phytases of the histidine acid phosphatase type (HAP) are commercially available. Most belong to the PhyA and PhyC families [6]. The founder of the PhyA family is PhyA phytase from Aspergillus niger; the other enzymes in this family come from moulds: (Aspergillus niger var. awamori) [7], Aspergillus fumigatus [8], Aspergillus terreus [9], Emericella rugulosa [10], Myceliophthora thermophila [9], and Talaromyces thermophiles [11]. These phytases have two optimal pH values (2.5 and 5.5) and an optimal temperature of 55–60 °C [12,13]. The molecular mass of unglycosylated PhyA phytases is predicted to be 48–50 kDa, although they are usually extensively glycosylated.

The PhyC family contains acid phytases from Escherichia coli [14,15] and Obesumbacterium proteus [16], as well as lysosomal acid phosphatases [17,18] and prostatic acid
phosphatases [19] from rats, humans, and other vertebrates. These phytases are intracellular proteins composed of 354–439 amino acids, with a molecular mass of ~42–45 kDa. These monomeric proteins are not N-glycosylated [13]. PhyC representatives typically have a single optimal pH (~5.0–6.0) and exhibit an optimal temperature of 40–60 °C.

The PhyA and PhyC families differ in their substrate specificity. PhyA is a so-called 3-phytase—the maximal reaction rate is achieved when the phosphate moiety in the third position of the phytate is removed. By contrast, members of the PhyC family exhibit so-called 6-phytase specificity, hydrolysing predominantly phosphate moieties in the sixth (1–4) position [6]. Enzymes from the PhyB and PhyD families are rarely used in practice.

Considering average daily weight gain (ADG), a comparison of diets containing phytases with phosphate-deficient diets indicated an unambiguous growth-stimulating effect of the enzymes during the entire period of the observation. However, comparison with a diet with adequate phosphorus revealed a high efficiency of the phytases only at early ages of the animals—the first month after weaning piglets from milk feeding [20]. As the slaughter time approaches, the efficiency of the mineral source of phosphorus in the diet is higher relative to the exogenous phytases. This drop in phytase efficiency can be prevented by increasing the phytase dosage. Both da Silva et al. [20] and Tsai et al. [5] reported that very high phytase dosages (3000–12,500 phytase activity units (FYT)/kg)—several times higher than dosages recommended by manufacturers—could adjust the ADG of the experimental group to the level of the group being fed a diet with adequate phosphorus or even somewhat surpass it. By contrast, standard phytase dosages (250–1000 FYT/kg) have no effect during this period, although they are efficient at an early age. When fed low-phytase diets during the finishing period, the slaughter weight of barrows was almost indistinguishable from that of animals fed a phosphorus-deficient diet. The efficiency of low phytase dosages in broiler feed is somewhat higher than in barrows [21]. One can speculate that this effect is due to elongation of the digestion time in the larger animals combined with insufficient stability of the exogenous phytases in the intestinal chyme. This conclusion leads to the hypothesis that using encapsulated phytase preparations that gradually release the enzyme from protective micro-containers—as a result of solubilising their surface coating—increases the enzyme efficiency.

The phytase of Obesumbacterium proteus (OPP) [16] is a typical representative of the PhyC family. It exhibits maximum activity at pH 4.9, thermal stability up to 60 °C (at pH 3.0) and a maximum activity of ~340 FYT/mg. There are reports about production of this enzyme in Pichia pastoris including its artificially modified derivative with enhanced thermal stability [22–24].

Isakova et al. [25] described an alternative approach to increasing the thermal stability of OPP. This enzyme was produced in the extremophile yeast Yarrowia lipolytica in a non-secreted (cytoplasmic) form. Encapsulating the enzyme within the yeast cell made it stable under short-term heating at 70–80 °C, the temperature required for spray drying feed premixes. Producing feed enzymes in a secreted form is a standard technological approach used by all manufacturers worldwide. It allows the production of pure enzyme preparations with high activity; they are usually added to mixed fodders in the course of the pelleting. This approach is advantageous for large manufacturers because it reduces the cost of logistics and warehousing. However, producing secreted enzymes has three major limitations—it requires high-quality raw matter for producing cultivation media, it requires utilisation of the cell mass as a waste and it requires high energy consumption during the vacuum concentration and spray drying stage. Y. lipolytica is well known for its ability to grow on fat-containing waste, converting it to highly valuable feed protein. Therefore, cultivation of the recombinant Y. lipolytica strains that produce OPP on fat-containing animal waste—as described by Isakova et al. [25]—would allow the simultaneous achievement of the following goals: (1) to produce a feed additive with high thermal stability (compatible with the spray drying of mixed fodder); (2) to utilise fat-containing waste without producing extra pollutants; (3) to reduce energy consumption for drying because vacuum concentration would be unnecessary; and (4) to enhance OPP stability in the stomach and
intestine of productive animals, with the gradual release of the phytase as it travels in the chyme through the intestine.

Taking into account the fact that [25] have reported the technology of obtaining microencapsulated phytase, including spray drying, and documented the fact of complete preservation of phytase activity at 75–80 °C during drying, which was not achievable when drying secretory phytase under similar conditions, the present work had an objective of demonstrating that microencapsulated phytase has a significantly higher ability to stimulate animal growth when added to feed than secretory phytase. To this end, a diet formula deficient in available phosphorous (0.15%) but with the total phosphorous including the grain phytate of 0.45% was developed to test the phytase effect on ADG in mice.

The experimental design of the study included three stages. First, a recombinant Y. lipolytica strain that produces OPP was cultivated on a pure swine blood sterilised by autoclaving, as described by [25]. The cell mass was then subjected to spray drying. Then the effect of the OPP-containing additive on the ADG of the mouse model was compared with a commercial phytase preparation—Ladozyme Proxi derived from Aspergillus ficuum (Emzyme, Ukraine) [11]. There were also control groups: a balanced diet with adequate phosphorus (PC, 0.21% available phosphorus and 0.54% total phosphorus), a balanced diet with insufficient phosphorus (NC), the NC diet supplemented with an inorganic phosphorus up to the adequate level (1.15% available phosphorus and 1.45% total phosphorus) and the NC diet supplemented with non-recombinant Y. lipolytica strain producing no phytase (mock additive) produced by cultivation on the swine blood and then subjected to spray drying. 2. Materials and Methods. The experimental group was fed the NC diet supplemented with 165 FYT/kg microencapsulated phytase, whereas the group of comparison was fed the same diet supplemented with 15,000 FYT/kg commercial secreted phytase.

2. Materials and Methods

2.1. Yeast Strains

Recombinant Y. lipolytica PO1f (pUV3-Op) producing OPP was described previously [25]. The parental strain of Y. lipolytica PO1f (MatA, leu2-270, ura3-302, xpr2-322, axp-2) was purchased from the CIRM-Levures collection (France), where it is deposited under registration number CLIB-724. The same strain is available from ATCC (registration number MYA-2613).

2.2. Preparing Feed Additives by Cultivation of the Yeast Strain

After storage at −70 °C, Y. lipolytica strains were cultivated on YPD complete agar medium (yeast extract (Difco), 10 g/L; Bacto Peptone (Difco), 20 g/L; glucose, 20 g/L; Bacto agar (Difco), 20 g/L) at 28 °C for 48 h. Both strains were passaged twice under the same conditions. After the third passage, the biomass was washed from a Petri dish (d = 90 mm) with 10 mL of sterile water and used as inoculum.

Two litres of whole swine blood were obtained from a slaughter-house (EVT, Kursk, Russia). The blood was separated into two 1 L containers, heated for 15 min in a boiling water bath to allow clotting and then homogenised by a hand blender equipped with a three-bladed propeller. The homogenised clot was dispensed into 750 mL Erlenmeyer flasks, with 100 mL of the homogenate in each. The flasks were closed with cotton stoppers and autoclaved at 121 °C for 30 min. In total, 20 flasks were prepared. Each flask was inoculated with a 5 mL suspension of PO1f (pUV3-Op) (10 flasks) or PO1f cells (10 flasks), placed in a microbiological shaker–incubator and incubated at 28 °C with shaking at 220 rpm.

After culturing PO1f (pUV3-Op) and PO1f, the cultures were pooled in a single vessel (one for each strain), adjusted to a volume of 2 L with sterile distilled water and subjected to spray drying at 70 °C by using the Anhydro PSBD 58 (Anhydro A/S, Denmark). The yield of the powder was ~60 g for each Y. lipolytica strain.
2.3. Determination of the Chemical Composition of the Swine Blood, Feed Additives Derived from It, and the Diets for the Mice

The chemical composition of the swine blood, feed additives derived from it, and the diets for the mice were determined at the Dokuchaev Soil Institute of the Russian Academy of Sciences by using energy dispersive X-ray fluorescence (EDXRF) analysis on the Respect instrument (Tolokonnikov Plant LLC, Moscow, Russia), as described previously [26].

2.4. Measurement of Phytase Activity

The phytase activity of the feed additives was measured by using a colourimetric assay for free phosphate ions released from sodium phytate as described previously [27]. Ten-milligram samples of each feed additive powder were placed in an Eppendorf tube. Then, 50 mg of glass beads (D = 0.3 mm) and 500 µL of 250 mM sodium-acetate buffer (pH 5.5) were added to each tube and incubated in an ice-cold bath for 10 min. The swollen cells were homogenised by stirring with a hand Vortex three times for 2 min each. The homogenates were clarified by centrifugation with a bench-top centrifuge at 12,000–14,000 rpm for 10 min.

The homogenates were diluted 2 and 4 times with 250 mM sodium-acetate buffer (pH 5.5), and 5 µL aliquots of each dilution (1, 2, and 4 times) were placed in the wells of a flat-bottom immunological plate (Nunc low-sorb, Denmark) containing 100 µL of substrate mix (5.1 mM sodium phytate in 250 mM sodium-acetate buffer, pH 5.5). The plate was closed to prevent exsiccation and incubated at 37 °C for 1 h. Then, 50 µL of Fiske and Subbarow reagent was added to each well. The plate was incubated for 10 min at room temperature and the absorbance at 415 nm was measured with a Uniplan plate spectrophotometer (Pikon, Russia). The phytase activity unit (FYT) is defined as a quantity of the enzyme releasing 1 µmol of the phosphate ion per minute under the above-mentioned conditions.

2.5. Diets for the Mice

The balanced PC diet contained corn grain (30%), sunflower grain with the shell (10%), forage wheat (40%), millet grain (15%) and oat (5%). The phosphate-deficient NC diet contained corn grain (50%), sunflower grain with the shell (25%), forage wheat (10%), millet grain (10%) and oat (5%). Both diets were composed from whole grains that had been ground in an electric coffee mill (3 min per portion).

The experimental (E) diet was prepared by supplementing 97 g of the NC diet with 3 g of the feed additive derived from the Y. lipolytica PO1f (pUV3-Op) strain. The mock additive (MA) diet was prepared by supplementing 97 g of the NC diet with 3 g of the feed additive derived from the Y. lipolytica PO1f strain. The phosphorus (P) diet was prepared by supplementing 97 g of the NC diet with 3 g of Na₂HPO₄·2H₂O. The commercial phytase (LP) diet was prepared by supplementing 97 g of the NC diet with 3 g of Ladozyme Proxi.

2.6. Animals

The experimental protocol was approved at a meeting of the Local Ethics Committee of the VIGG (Protocol No. 1 dated 26 June 2019). Mongrel white mice were bred in the vivarium of the Skryabin Academy of Veterinary Medicine and Biotechnology. The mice were introduced to the experiment once they weighed 11.0 ± 0.3 g, regardless of their age (typically the fifth day after weaning from the dam). The mice from the same brood were randomly distributed to different experimental groups. Each group was composed of animals introduced to the experiment gradually as they reached the specified body weight. Each group contained five males and five females kept in the same cage. After weaning and until introducing the mice to the experiment, they were fed with the PC diet. After introduction in the experiment, they were fed on the experimental diets for 14 days. The mice were given food twice per day. Each mouse was weighed daily before the second feeding. The mice had ad libitum access to water and were kept on a 12 h photoperiod.
2.7. Measuring ADG and Statistical Analysis

ADG was calculated for each group for days 5, 10 and 14 after the start of the experiment (day 0). The standard error of the mean (SEM) was calculated as described previously [28]. The Mann–Whitney U test with the minimal thresholds of Fisher’s coefficient was used to determine differences between the experimental groups (E, MA and P) and the control groups (PC and NC) for days 5, 10 and 14 (p < 0.05). Statistics 8.0 for Windows software was used for the statistical analysis. In addition, the relative ADG for each experimental group was calculated for the PC and NC groups at days 5, 10 and 14 of the experiment.

3. Results

3.1. Manufacturing Feed Additives by Using Y. lipolytica Strains

The whole swine blood without separation into liquid and solid fractions was used as a substrate for cultivation of the Y. lipolytica PO1f and PO1f (pUV3-Op) strains. The obtained additives were used to prepare diets for the mice. The additives derived from the Y. lipolytica PO1f and PO1f (pUV3-Op) strains cultivated on swine blood were administered at 3% of the diet weight. This is the maximum ratio of the dry yeast (both Y. lipolytica and Saccharomyces cerevisiae) recommended for producing mixed fodder [29]. Considering that the specific phytase activity in the powder of PO1f (pUV3-Op) additive after spray drying was 55 FYT/g, the exogenous phytase activity in the group E diet was 165 FYT/kg, which is 2–3 times less than recommended by most manufacturers [5,20,21,30]. The Ladozyme Proxi commercial phytase was also administered at 3% of the diet weight. As a result, the specific phytase activity in the LP diet was 15,000 FYT/kg. This is 30 times greater than recommended by the manufacturer. Such a high concentration of the commercial phytase preparation was chosen to achieve the maximum theoretically possible physiological effect on ADG in vivo. The final composition of all experiment diets is shown in Table 1.

Table 1. Chemical composition of the diets used for feeding mice in the experimental groups. All values except moisture are normalised per dry mass.

| Nutritional Parameters, % | PC Positive Control | NC Negative Control | NC Supplemented with 2% Na₂HPO₄ 2H₂O |
|---------------------------|--------------------|---------------------|-------------------------------------|
| Moisture                  | 16.2               | 15.2                | 14.7                                |
| Total phosphorus          | 0.54               | 0.45                | 1.45                                |
| Accessible phosphorus     | 0.21               | 0.15                | 1.15                                |
| Na                        | 0.01               | 0.02                | 0.02                                |
| Mg                        | 0.29               | 0.27                | 0.26                                |
| Al                        | 0.05               | 0.1                 | 0.1                                 |
| Si                        | 0.62               | 0.29                | 0.28                                |
| S                         | 0.16               | 0.14                | 0.14                                |
| Cl                        | 0.05               | 0.05                | 0.05                                |
| K                         | 0.68               | 0.57                | 0.55                                |
| Ca                        | 0.11               | 0.09                | 0.09                                |

Abbreviations: NC, balanced diet deficient in phosphorus; PC, balanced diet with adequate phosphorus; P, phosphorus.

3.2. Testing the Impact of the Feed Additives on ADG

The reliability of data regarding the impact of the phytase administered in the diet on the ADG depended substantially on individual differences in the average body weight of the mice and the irregularity of their daily weight gain. Preliminary observations revealed the occurrence of clear distinguishable leaders and outsiders in each brood that differed substantially in body weight dynamics (data not shown). We found that individual differences manifested most clearly during a lag period of variable duration until the body weight of the weanlings reached ~11 g. At this time, all animals gained weight...
almost linearly until reaching ~18 g. Subsequently, the individual differences became critical again. Taking this observation into consideration, we employed a gradual (not simultaneous) scheme of introducing the animals to the experiment. The weanlings from several available broods were fed on a balanced diet (PC) and were weighed daily; each mouse was introduced to the experiment (PC diet was changed to an experimental diet) once they reached 11 g in weight. Each brood was distributed randomly among the experimental groups. Then, the animals were kept on one of the six experimental diets for 14 days and then withdrawn from the experiment. Each mouse was weighed daily during the entire observation period (Figure 1).

![Figure 1](image_url)

**Figure 1.** Dynamics of the live body weight of the mice fed with the different experimental diets (six animals per group): group P (mineral phosphorus); group E (encapsulated phytase of Obesumbacterium proteus (OPP), derived from the recombinant Yarrowia lipolytica PO1F (pUV3-Op) strain); group LP (commercial Ladozyme Proxi soluble phytase) and group MA (mock additive derived from the non-recombinant Y. lipolytica PO1F strain). Each growth curve is shown in the same graph with the balanced diet deficient in phosphorus (NC) and the balanced diet with adequate phosphorus (PC).

The proposed method allowed for minimisation of the bias due to individual features of the animals in the ADG, permitted determination of SEM in each group and allowed an accurate check of the null hypothesis that there were no differences between the groups for ADG by using the Mann–Whitney U test. Table 2 presents the data of the comparison of ADG in the different experimental groups.
Table 2. Comparison of ADG in the different experimental groups of the mice. The $p$ values are based on the Mann–Whitney U test.

| Group | Mean ADG ± SEM of ADG | % ADG Relative to | $p$  | Mean ADG ± SEM of ADG | % ADG Relative to | $p$  | Mean ADG ± SEM of ADG | % ADG Relative to | $p$  |
|-------|-----------------------|-------------------|------|-----------------------|-------------------|------|-----------------------|-------------------|------|
|       | Day 5                 | Day 10            | Day 14          |                      | Day 5                 | Day 10            | Day 14          |                      | Day 5                 | Day 10            | Day 14          |
| PC    | 0.59 ± 0.29           | -                 | 34.09           | - p < 0.05           | 0.66 ± 0.14  | 24.73           | - p < 0.05       | 0.65 ± 0.25  | 24.47           | - p < 0.05       |
| NC    | 0.44 ± 0.19           | -                 | 25.42           | - p < 0.05           | 0.53 ± 0.26  | -19.83          | - p < 0.05       | 0.52 ± 0.29  | -19.65          | - p < 0.05       |
| E     | 0.53 ± 0.20           | -9.15             | 21.81           | - p > 0.05           | 0.60 ± 0.18  | -8.18           | 14.52           | - p > 0.05     | 0.58 ± 0.18  | -10.33           | 11.61           | - p > 0.05     |
| MA    | 0.51 ± 0.09           | -13.55            | 15.91           | - p > 0.05           | 0.54 ± 0.26  | -17.41          | 2.26            | - p > 0.05     | 0.52 ± 0.31  | -18.67           | 1.23            | - p > 0.05     |
| P     | 0.60 ± 0.19           | 1.69              | 36.36           | - p > 0.05           | 0.63 ± 0.30  | -3.64           | 20.18           | - p > 0.05     | 0.64 ± 0.35  | -1.65            | 22.40           | - p > 0.05     |
| LP    | 0.48 ± 0.20           | -17.96            | 10.00           | - p > 0.05           | 0.52 ± 0.17  | -20.28          | -0.56           | - p > 0.05     | 0.55 ± 0.17  | -15.48           | 5.19            | - p > 0.05     |

Abbreviations: ADG, average daily weight gain; E, experimental diet containing the feed additive derived from the Yarrowia lipolytica PO1f (pUV3-Op) strain; LP, diet containing the Ladozyme Proxi additive; MA, diet containing the feed additive derived from the Y. lipolytica PO1f strain; NC, balanced diet deficient in phosphorus; P, diet containing Na$_2$HPO$_4$·2H$_2$O; PC, balanced diet with adequate phosphorus; SEM, standard error of the mean; % ADG relative to change in ADG relative to the PC or NC diet.
4. Discussion

The practical applicability of the microencapsulation technology within yeast cells, described previously [25], depends on the availability of affordable substrates for cultivation of the producer strain, and not only on the yield of the enzyme. *Y. lipolytica* is known for its ability to reduce the peroxide index of the substrate and its overall detoxification of the different types of organic waste [31–33].

The yield of the active enzyme in microencapsulated form at the end of fermentation is usually significantly inferior to the yield of the secretory enzyme. However, this is compensated by the low cost of raw materials for cultivating yeast strains, and the waste-free manufacturing circle and energy savings at the drying stage (a microencapsulated enzyme, unlike a secretory one, can be concentrated by centrifugation instead of membrane filtration or vacuum evaporation followed by spray drying). We also assume that microencapsulation of the enzyme can significantly increase its biological activity within the feed additive per unit of activity, since such an enzyme, unlike the secretory one, will not lose activity when passing through the stomach. We also assume that the microencapsulated enzyme will more efficiently cleave the phytate released from the grain in the intestine, since the processes of release of phytate from the swelling grain in the duodenum and the release of phytase from microcapsules will proceed in parallel, and the phytase will not be degraded by pancreatic proteases while the phytate is still spatially isolated from it by seed shells.

Swine blood is a massive type of the organic waste is rich in fat. To the best of our knowledge, swine blood has never been previously reported as a substrate for cultivation of *Y. lipolytica*. It can be hypothesized that it can be applicable for this; however, its nutritional value as a feed additive is limited because it contains an excess of iron, which is unfavourable for growth of the microorganisms. This was confirmed with a low yield of the specific activity 360 FYT/L. In comparison to the reported activity of OPP 340,000 FYT/L produced by the same strain PO1f (pUV3-Op) in a medium with peptone and glucose [25], this is an extremely low yield.

The yield of the wet biomass was about 200 g/L of the wet cells together with the residue of the blood clot that was converted to the powder with the final yield of 30 g from 1 L of the culture. This powder after spray drying at 70 °C had activity 55 FYT/g. As far as the maximum specific activity of the pure OPP is 310 FYT/mg of protein [16], this means that the powder contained ~0.05% of the active enzyme of the interest. The loss of the specific activity after the spray drying was 8.3%.

Biological trials of feed phytase preparations are usually carried out on broilers and pigs. This approach allows a complex analysis of the physiological activity of phytase preparation on carcass parameters; relative weights of visceral organs; meat quality parameters; phosphorous content in excreta [21]; length of the bones [5]; relative yield of the carcass and the meat [20]; physical, mechanical and chemical properties of the tibia; and morphology of the jejunum [30]. These parameters are highly relevant to the practical efficiency of phytase additives; however, using productive animals as a physiological model is resource-intensive—in terms of labour, time and cost. Hence, biological trials using pigs or broilers are rarely used for preliminary trials of enzyme preparations until they are adopted for commercial manufacturing.

Using mice for experiments is fast and inexpensive compared with productive animals. This approach is accessible for laboratory-scale testing of the efficiency of phytase preparations as well as other enzyme-containing feed additives. However, there are only a few published examples of using mice for similar purposes [28]. Measuring daily weight gain in mice has been applied to determine unfavourable side effects of immunisation with *Streptococcus bovis* inactivated or attenuated vaccines [34] and for testing whether new medicines decrease caloric intake and weight gain [35]. The described models use adult mice or, at best, animals with a weight of ~18 g, that have almost completed growing and therefore have limited utility as a model for ADG measurement in productive animals. Taking into account the absence of adequate descriptions of a mouse model for determina-
tion of the phytase impact on the ADG in the published reports, we undertook an original approach for this task.

The proposed model of testing the ADG by using mice was found to be relevant. This was confirmed by the fact there were consistent differences in ADG between the PC and NC groups that were significant for the second threshold at day 5 and for the third threshold at days 10 and 14 of the experiment. By complementing the NC diet with the mineral phosphorus supply (P diet), ADG was indistinguishable from the PC diet at days 5, 10 and 14 of the experiment. The difference between the NC and PC groups was reliable for the first threshold at days 5 and 10 and for the second threshold at day 14.

Testing the commercial phytase Ladozyme Proxi (LP diet) administered in a traditional soluble form in 30-fold excess over the dosage recommended by the manufacturer showed no difference compared with the NC diet, but it did differ significantly compared with the PC diet for the third threshold at days 10 and 14. These findings indicate that the commercial phytase has no effect on ADG of the proposed mouse model. By contrast, the encapsulated OPP provided a reliable, statistically significant effect on ADG. There was no difference compared with the PC diet at days 5 and 10, with a difference for the first threshold at day 14. There were differences between the E and NC diets for the first threshold on days 10 and 14. The absence of differences between the E and either the PC or NC diets at day 5 could be explained with a relatively high average ADG in all groups at this time (based on the SEM). The impact of OPP cannot be explained by presence of Y. lipolytica and the rest of the whole blood substrate in the diet because the diet with an additive derived from the non-recombinant Y. lipolytica (MA group) exhibited no differences compared with the NC group but differed from the PC group for the second threshold at day 10 and for the third threshold at day 14.

5. Conclusions

Our data confirm a high impact of the encapsulated OPP on ADG in the proposed mouse model. The feed additive produced by fermentation of whole swine blood exhibited high efficiency regardless of the relatively low phytase activity. Administration of this additive at a dosage of 165 FYT/kg impacted ADG more than the commercial phytase Ladozyme Proxi, even though the commercial formulation was provided as a 30-fold overdosage. One can hypothesise that the encapsulated phytase has a greater effect in animals with a large body size that are not susceptible to the administration of traditional PhyA or PhyC phytases.

Author Contributions: M.A.D. manufactured the encapsulated the phytase specimens and conducted the animal experiments. E.Y.E. carried out the genetic engineering manipulations and selected the Y. lipolytica recombinant strain. E.V.T. carried out statistical analysis of the data. A.B.S. conceived and designed the research and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Adeola, O.; Cowieson, A.J. BOARD-INVITED REVIEW: Opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. *J. Anim. Sci.* 2011, 89, 3189–3218. [CrossRef] [PubMed]

2. Cosgrove, D.J. The chemistry and biochemistry of inositol polyphosphates. *Rev. Pure Appl. Chem.* 1966, 16, 209–215.

3. Morgan, N.; Walk, C.; Bedford, M.; Burton, E. Contribution of intestinal- and cereal-derived phytase activity on phytate degradation in young broilers. *Poult. Sci.* 2015, 94, 1577–1583. [CrossRef] [PubMed]

4. National Research Council. *Nutrient Requirements of Swine: Eleventh Revised Edition*; The National Academies Press: Washington, DC, USA, 2012; ISBN 978-0-309-48903-4.

5. Tsai, T.C.; Dove, R.; Bedford, M.R.; Azain, M.J. Effect of phytase on phosphorous balance in 20-kg barrows fed low or adequate phosphorus diets. *Anim. Nutr.* 2020, 6, 9–15. [CrossRef] [PubMed]

6. Oh, B.-C.; Choi, W.-C.; Park, S.; Kim, Y.-O.; Oh, T.-K. Biochemical properties and substrate specificities of alkaline and histidine acid phytases. *Appl. Microbiol. Biotechnol.* 2004, 63, 362–372. [CrossRef] [PubMed]

7. Piddington, C.S.; Houston, C.S.; Palloheimo, M.; Cantrell, M.; Miettinen-Oinonen, A.; Nevalainen, H.; Rambosek, J. The cloning and sequencing of the genes encoding phytase (*phy*) and pH 2.5-optimum acid phosphatase (*aph*) from *Aspergillus niger* var. *avamori*. *Gene* 1993, 133, 55–62. [CrossRef]

8. Pasamontes, L.; Haiker, M.; Wyss, M.; Tessier, M.; van Loon, A.P. Gene cloning, purification, and characterization of a heat-stable phytase from the fungus *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* 1997, 63, 1696–1700. [CrossRef]

9. Mitchell, D.B.; Vogel, K.; Weimann, B.J.; Pasamontes, L.; van Loon, A.P.G.M.Y. The phytase subfamily of histidine acid phytases: Isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*. *Microbiology* 1997, 143, 245–252. [CrossRef]

10. Yadav, R.S.; Tarafdar, J. Phytase and phosphatase producing fungi in and semi-arid soils and their efficiency in hydrolyzing different organic P compounds. *Soil Biol. Biochem.* 2003, 35, 745–751. [CrossRef]

11. Pasamontes, L.; Haiker, M.; Henriquez-Huecas, M.; Mitchell, D.B.; van Loon, A.P. Cloning of the phytases from *Emericella nidulans* and the thermophilic fungi *Talaromyces thermophila*. *Biochim. Biophys. Acta.* 1997, 1353, 217–223. [CrossRef]

12. Ullah, A.H. *Aspergillus fuscus* ficuum phytase: Partial primary structure, substrate selectivity, and kinetic characterization. *Prep. Biochem.* 1988, 18, 459–471. [CrossRef] [PubMed]

13. Wyss, M.; Brugger, R.; Kronenberger, A.; Rémy, R.; Fimbel, R.; Oesterhelt, G.; Lehmann, M.; van Loon, A.P. Biochemical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): Catalytic properties. *Appl. Environ. Microbiol.* 1999, 65, 367–373. [CrossRef]

14. Dassa, E.; Boquet, P.L. Identification of the gene appA for the acid phosphatase (pH optimum 2.5) of *Escherichia coli*. *Mol. Gen. Genet.* 1985, 200, 68. [CrossRef] [PubMed]

15. Dassa, J.; Garraud, J. The complete nucleotide sequence of the *Escherichia coli* gene appA reveals significant homology between pH 2.5 acid phosphatase and glucose-1-phosphatase. *J. Bacteriol.* 1990, 172, 5497–5500. [CrossRef] [PubMed]

16. Zinin, N.V.; Serkina, A.V.; Gelfand, M.S.; Shevelev, A.B.; Sineoky, S.P. Gene Cloning, Expression and characterization of novel phytase from *Obesumbacterium proteus*. *FEMS Microbiol. Lett.* 2004, 236, 283–290. [CrossRef] [PubMed]

17. Pohlmann, R.; Krentler, C.; Schmidt, B.; Schröder, W.; Lorkowski, G.; Culley, J.; Mersmann, G.; Geier, C.; Waheed, A.; Gottschalk, S. Human lysosomal acid phosphatase: Cloning, expression and chromosomal assignment. *EMBO J.* 1988, 7, 2343–2350. [CrossRef]

18. Geier, C.; Figura, K.V.; Pohlmann, R. Molecular cloning of the mouse lysosomal acid phosphatase. *Biochim. Biophys. Acta.* 1991, 109, 301–304. [CrossRef]

19. Van Etten, R.L.; Davidson, S.E.; MacArthur, H.; Moore, D.L. Covalent structure, disulfide bonding, and identification of reactive surface and active site residues of human prostatic acid phosphatase. *J. Biol. Chem.* 1991, 266, 2313–2319. [CrossRef]

20. Da Silva, C.A.; Callegari, M.A.; Dias, C.P.; Bridi, A.M.; Poppe, L.; Martins, C.C.D.S.; Dias, F.T.F.; Passos, A.; Hermes, R. Increasing doses of phytase from *Citrobacter braakii* in diets with reduced inorganic phosphorus and calcium improve growth performance and lean meat of growing and finishing pigs. *PLoS ONE* 2019, 14, e0217490. [CrossRef] [PubMed]

21. Srikanthithasan, K.; Macelline, S.P.; Wickramasuriya, S.S.; Tharanangi, H.; Jayasena, D.D.; Ho, J.-M. Effects of adding phytase from *Aspergillus niger* to a low phosphorus diet on growth performance, tibia characteristics, phosphorus excretion, and meat quality of broilers 35 days after hatching. *J. Poult. Sci.* 2020, 57, 28–36. [CrossRef]

22. Gordeeva, T.; Borschevskaya, L.; Kalinina, A.N.; Sineoky, S.; Voronin, S.P.; Kashirskaya, M.D. Expression and characteristics of phytases from *Obesumbacterium proteus* in *Pichia pastoris* yeast. *Biotechnologiya* 2018, 34, 18–25. [CrossRef]

23. Gordeeva, T.; Borschevskaya, L.; Kalinina, A.N.; Sineoky, S.; Kashirskaya, M.D.; Voronin, S.P. Increase in thermal stability of phytase from *Citrobacter freundii* by site-directed saturation mutagenesis. *Biotechnologiya* 2018, 34, 33–42. [CrossRef]

24. Gordeyeva, T.L.; Borschevskaya, L.N.; Kalinina, A.N.; Sineoki, S.P.; Voronin, S.P.; Kashirskaya, M.D. Recombinantnyy produtsent kormovogo fermenta fitazy na osnove drozhdzhey *Pichia pastoris*. *Aktual’nyaya Biotechnol.* 2018, 3, 117.

25. Isakova, E.P.; Serdyuk, E.G.; Gessler, N.N.; Trunbikova, E.V.; Biryukova, Y.K.; Epova, E.Y.; Deryabin, Y.I.; Nikolaev, A.V. A new recombinant strain of *Yarrowia lipolytica* producing encapsulated phytase from *Obesumbacterium proteus*. *Dokl. Biochem. Biophys.* 2018, 481, 201–204. [CrossRef] [PubMed]

26. Savichev, A.T.; Sorokin, S.E. Rentgenofluorescentnyy energodispersionnyy analiz zol’nykh elementov v rasteniakh. *Agrokhimiya* 2001, 12, 61–67.

27. Fiske, C.H.; Subbarow, Y. The colorimetric determination of phosphorus. *J. Biol. Chem.* 1925, 66, 375–400. [CrossRef]
28. Norton, J.D.; Yang, S.P.; Diffley, P. Influence of source and quantity of protein on the development of immunity and resistance to African trypanosomiasis. *Infect. Immun.* 1986, 51, 455–460. [CrossRef]

29. Czech, A.; Smolczyk, A.; Grela, E.R.; Kiesz, M. Effect of dietary supplementation with *Yarrowia lipolytica* or *Saccharomyces cerevisiae* yeast and probiotic additives on growth performance, basic nutrients digestibility and biochemical blood profile in piglets. *J. Anim. Physiol. Anim. Nutr.* 2018, 102, 1720–1730. [CrossRef]

30. Ziarat, M.; Kermanshahi, H.; Mogaddam, H.; Majidzadeh Heravi, R. Performance of an *Escherichia coli* phytase expressed in *Lactococcus lactis* on nutrient retention, bone traits and intestinal morphology in broiler chickens. *J. Anim. Physiol. Anim. Nutr.* 2020, 104, 909–917. [CrossRef]

31. Ali, S.S.; Al-Tohamy, R.; Koutra, E.; El-Naggar, A.H.; Kornaros, M.; Sun, J. Valorizing lignin-like dyes and textile dyeing wastewater by a newly constructed lipid-producing and lignin modifying oleaginous yeast consortium valued for biodiesel and bioremediation. *J. Hazard. Mater.* 2021, 403, 123575. [CrossRef] [PubMed]

32. Quarterman, J.C.; Slininger, P.J.; Hector, R.E.; Dien, B.S. Engineering *Candida phangngensis*—an oleaginous yeast from the *Yarrowia* clade—for enhanced detoxification of lignocellulose-derived inhibitors and lipid overproduction. *FEMS Yeast Res.* 2018, 18, foy102. [CrossRef] [PubMed]

33. Iwama, R.; Kobayashi, S.; Ishimaru, C.; Ohta, A.; Horiuchi, H.; Fukuda, R. Functional roles and substrate specificities of twelve cytochromes P450 belonging to CYP52 family in n-alkane assimilating yeast *Yarrowia lipolytica*. *Fungal Genet. Biol. FG B* 2016, 91, 43–54. [CrossRef] [PubMed]

34. Paiva, A.D.; Fernandes, K.M.; Dias, R.S.; Rocha, A.S.; de Oliveira, L.L.; Neves, C.A.; de Paula, S.O.; Mantovani, H.C. Effects of the oral administration of viable and heat-killed *Streptococcus bovis* HC5 cells to pre-sensitized BALB/c mice. *PLoS ONE* 2012, 7, e48313. [CrossRef] [PubMed]

35. White, H.M.; Acton, A.J.; Considine, R.V. The Angiogenic inhibitor TNP-470 decreases caloric intake and weight gain in high-fat fed mice. *Obesity Silver Spring MD* 2012, 20, 2003–2009. [CrossRef] [PubMed]