Investigation on bioavailability of rumen bacterial and protozoal phosphorus using a chick-model

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Abstract Ruminal bacteria and protozoa provide excellent organic phosphorus (P) sources for the host animal. This experiment was conducted to compare the bioavailability of bacterial and protozoal P in comparison with that of dicalcium phosphate (DCP) as a reference phosphorus source. Bacteria and protozoa were isolated from the rumen contents of dairy cows and the chick-model was used to determine the phosphorus bioavailability. Fifteen day-old chicks were used in a completely randomized design with 4 treatments consisting of a control diet (without P, as a basal diet), bacterial P+basal diet, protozoalP+basal diet, and DCP+basal diet. Phosphorus balance was selected as the response criterion for phosphorus bioavailability assessment, using multiple linear regressions. The results showed a higher phosphorus bioavailability of protozoal P and DCP compared with bacterial P ($P<0.05$); with no significant difference between protozoal P and DCP. Relative phosphorus bioavailability values of bacterial source ranged from 0.451 to 0.495, using protozoal source as reference. Bioavailability of P in rumen protozoa, relative to that in DCP, was high (approximately 97%); therefore rumen protozoa may serve as an important source of organic P in ruminants for providing a significant portion of phosphorus requirements.

Keywords: Rumen bacteria, rumen protozoa, phosphorus, bioavailability, chick-model, dairy cows

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Introduction

Organic and inorganic minerals are usually absorbed with different mechanisms in the small intestine which may increase their biological availability. Therefore, utilization of organic phosphorus (P) may lead to decreased use of inorganic P supplements, which will not only decrease the total amount of P excreted but also the potentially more polluting water soluble P (Kebreab et al., 2008). Phosphorus in the rumen microorganisms is a constituent of primary cell metabolites such as nucleotides, coenzymes, teichoic acids of the cell walls of Gram-positive bacteria and phospholipids, which occur mainly in the cytoplasmic and outer membranes of Gram-negative bacteria (Durand and Kawashima, 1980). On the other hand, microorganisms are capable of accumulating excess inorganic phosphate in the form of inorganic polyphosphate (Kulaev et al., 1999). It appears that ruminal microorganisms are largely dependent on dietary phosphorus for their P needs (Durand and Kawashima, 1980) and the factors which stimulate microbial growth (e.g., digestible organic matter) would increase phosphorus uptake by rumen microbes that is consequently passed to the lower tract for utilization by the host animal (Bravo et al., 2003).

Environmental concerns with phosphorus (P) have forced the animal industry to re-evaluate the levels formulated in diets. It has been demonstrated in numerous research trials that excess P intake equates to excess P out in the manure. The ideal way of controlling P intake is developing diets that closely match the animal's requirement and implementing feeding management practices to ensure those levels are being consumed. In this regards, a better understanding of the bioavailability of phosphorus in rumen bacteria and protozoa is essential so that diets can be formulated to meet minimum requirements without over supplementing phosphorus and causing environmental problems.

Therefore, objectives of this study were 1) to evaluate of availability of P content in ruminal bacterial and protozoal as a two main phosphorus sources using chick-model and 2) to compare DCP, as a P supplement usually used in animal diets, with bacterial and protozoal P sources.

Materials and methods

Phosphorus source preparations

Rumen bacteria

Rumen fluid was collected from three dry Holstein cows fed at energy maintenance level with a diet containing hay, wheat straw and concentrate mix in the proportion of 0.47, 0.22 and 0.31 on as fed basis, respectively. Rumen bacteria were isolated using a modified procedure as described by Pathak et al. (1996). After collection, about 20 liters of fluid, needed to obtain sufficient bacterial number, was filtered through two layers of surgical gauze and transported to the laboratory in pre-heated thermo flasks (about 39°C) and left aside about 45 minutes in order to deposit of feed residues and very large protozoa. The extract was then transferred into 250- milliliter centrifuge containers and centrifuged at 1000 × g and 4°C for 5 minutes. The supernatant was evacuated and collected carefully and centrifuged at 17000 × g and 4°C for 20 min (Beckman Avanti®-High speed Centrifuge). The supernatant was evacuated and outcome depositions were mustered. Because of large volume of rumen extract needed (about 20 liters), these procedures were repeated several times and final depositions containing rumen bacteria were transferred to special freeze-dryer dishes and kept at -80°C temperature for 43 hours. Bacterial samples were freeze-dried (CHRiST®) and milled through a 0.5 mm screen on a hammer mill (Retsch®) and stored under vacuum.

Rumen protozoa

About 30-40 liters of rumen fluid were obtained 3 to 4 hours post feeding from four dry Holstein cows. Rumen protozoa were isolated using a modified Pathak et al. (1996) procedure. The rumen fluid was filtered through ten layers of surgical gauze and transported to the laboratory in pre-heated thermo flasks (about 39°C) and left aside, allowing large protozoa to precipitate as a visible white layer at the bottom of funnel shape vessels. This layer was withdrawn carefully and collected in a beaker and kept at 4°C for later stages. The supernatant was transferred to 250- milliliter centrifuge tubes and centrifuged at 500 × g and 4°C for 15 min (Eppendorf® Multi-Propose Centrifuge). The grey-white layer at the bottom of centrifuge tubes was collected and added to the beaker containing protozoa and kept at 4°C. After being centrifuged, contents of the beaker were transferred to special freeze- dryer dishes and kept at -80°C temperature for 48 hours. Freeze-drying and milling procedures were similar to that used for bacterial preparation.

Dicalcium phosphate (DCP)

Commercial dicalcium phosphate was used as a reference inorganic phosphate source (Table 1).

Table 1.Commercial dicalcium phosphate characteristics

Item	Composition (%)
Phosphorus	17
Calcium	23
Flouride	0.17
Water solubility	85
Dry matter	97
pH	3.76

Chicken preparation and training

Thirty 1-day-old commercial broiler chickens (Ross, Iranian agency) were housed in floor pens containing litter composed of wood shavings and received a corn-based starter diet (Table 2). At 12 days of age, 24 chicks were selected and were trained for forced feeding by using a funnel (6-mm outside diameter) as described by Bilgili et al. (1982). During this period all birds were housed in layer cages with mesh bottoms to adapt to conditions during the digestion trial. No abnormality was shown by chicken during this period.

Table 2. Ingredients and nutrient composition of the starter diet.

	Dietary components,%
Ingredients	
Corn	56.53
Soybean	37.29
Oil	2.53
Dicalcium phosphate	1.53
Iodized salt	0.36
Shell powder	1.26
Mineral supplement[1]	0.25
Vitamin supplement[2]	0.25
Nutrient composition[3]	
ME (kcal/kg)	2950
Crude protein (%)	21.4
Calcium (%)	0.93
Total P (%)	0.68
Non-phytate P (%)	0.44
Methionine (%)	0.36
Lysine (%)	1.23

[1] magnesium, 99.2; zinc, 84.7 mg; iron, 50 mg; copper, 10 mg; iodine, 0.99 mg; selenium, 0.2 mg (supplied per kilogram of diet).
[2] vitamin A, 9,000 IU; cholecalciferol, 2,000 IU; vitamin E, 18 mg; riboflavin, 6.6 mg; choline chloride, 250 mg; vitamin B_{12}, 15 µg; vitamin B_6, 2.94 mg;biotin, 0.11 mg; folic acid, 1 mg; vitamin B_5, 29.65 mg; vitamin B_3, 9.8 mg; vitamin B_1, 1.75 mg; vitamin K_3, 2 mg.
[3]Calculated according to NRC (1994) from the diet composition.

Diets

Four diets were prepared by mixing corn starch as a basal diet containing 0.4% phosphorus as recommended by NRC (1994) for chicken at starter stage. In control treatment, diet contained only corn starch, which was milled through a 1- mm screen on a hammer mill. Control treatment was selected for determining the endogenous phosphorus loss. Bacterial treatment consisted of corn starch plus dried rumen bacterial to provided 0.4% total phosphorus content. Bacterial, protozoal and DCP treatments consisted of corn starch plus dried rumen bacteria, dried rumen protozoa and DCP, respectively to provided 0.4% total phosphorus. Each diet was mixed well, weighted (5 g) by 3 digit balance, kept in air tight plastic bags and stored at 4°C.

Digestion trial

A digestion trial was performed using forced- fed broiler chickens from 15 to 21 days of age. Twenty four birds (6 replications in each treatment) were housed in individual layer cages with mesh bottoms. Digestion period consisted of 4 days of adaptation, followed by 72 hr forced- feeding (Sibbald, 1979) with 5 g of feed from each treatment 4 times at 6- hr intervals (20g/day). Birds had free access to water from 30 min after being force- feeding to prevent regurgitation of feed from the crop). The cages were kept in controlled a room (28°C and approximately 58% ± 3 relative humidity). Excreta were collected for each 24-hour period at days 19, 20 and 21. Contaminants, such as down and scales, were carefully removed, and excreta were stored in containers at –25°C. Total excreta from each chick were subsequently dried in an oven at 70°C, weighed, ground through a 0.5- mm sieve, and stored in an airlock plastic vessel at 4°C until analysis.

Chemical analysis

The P contents of the basal diet (corn starch), phosphorus sources and excreta were analyzed by spectrophotometric procedures (AOAC, 2002).

Statistical Analyses

The data were analyzed using the general linear model procedure, and if a significant (P<0.05) effect was was detected the means were compared by the Duncan's test (SAS, 2005). Relative bioavailability (RBV) of microbial P was tested by the procedure described by Littell et al., (1997). In the SAS statements, the SOLUTION option was invoked in the MODEL statement to obtain printed estimates of the regression parameters because a CLASS term was used in the GLM statements. The I (which stands for "inverse" of the X´X matrix) option was used to obtain the covariance information needed to construct a confidence interval for RBV.

Results and Discussion

Chick-model vs. other species for study of bioavailability

In the present study chick-model was used for estimation of P bioavailability. The main species used as a model are chickens, rats, pigs, guinea pigs and primates. For estimation of P availability in rumen microbes using small samples the large species are not suitable. Rats seem to be similar to dairy cows but, the physiological characteristics of the rat also make it an unsuitable model for research on P bioavailability (Gueguen et al., 2000). Unlike chicks and cows, the rat intestine contains high levels of phytase activity enabling it to hydrolyze phytates. Furthermore, chickens kept for this purpose may be less expensive to maintain than multicannulated cattle. Pfeffer et al. (1970) showed that in ruminants as in monogasteric species, the small intestine are the major site of P absorption.

Phosphorus digestibility

Characteristics of phosphorus source in different treatments are shown in Table 3. The results of phosphorus digestibility for each phosphate sources are shown in Table 4.

Chick on control treatment showed negative digestibility. The loss of P from the body in chicks on the P-free diet is representative of the endogenous and metabolic P. Data on endogenous and metabolic P loss in broiler chickens are scarce, but estimates have generally ranged from 250 to 450 mg of endogenous P/kg of DM intake (Rutherfurd et al., 2004). The endogenous loss is assumed to be constant under P deficient conditions and is usually expressed on a live weight basis.

Table 3. Composition (mg/g) of phosphorus sources from bacteria, protozoa and dicalcium phosphate (DCP).

Source	Phosphorus	Nitrogen	Nitrogen/Phosphorus
Rumen bacteria	13.85	71.10	5.13
Rumen protozoa	29.70	55.30	1.86
DCP	170.00	-	-

Martz et al. (1990, 1999) estimated endogenous P to be 900 to 1500 mg/100 kg body weight (BW) per day in lac tating cows and 700 mg/100 kg BW per day in non-lactating cows using ^{32}P. Using extrapolation to zero Pintake, Conrad (1999) obtained 3900 mg/d, or 700 mg/100 kg BW (assuming 600 kg BW) in lactating cows. The values used by different feeding standards range from 1000 to 3500 mg/100 kg of live weight (Tamminga, 1992). In the present study, endogenous and metabolic P loss was estimated at 413 mg/kg DM intake or 27.6 mg/kg body weight, which is within the range of reported values. The endogenous and metabolic P loss in chickens in control treatment was used to adjust true P digestibility in other treatments.

The P source affected the true P digestibility. Both P from rumen protozoa and DCP showed highest true digestibility and P from rumen bacterial showed lowest digestibility (P<0.05). However, phosphorus true digestibility did not differ significantly between protozoal and DCP treatments (P>0.05).

The following models were used to estimate P absorbability (Y) :

$$Y_{Protozoa} = 1.44 \times 10^{-7} + 0.945\ P \quad (1)$$

$$Y_{Bacteria} = 1.44 \times 10^{-7} + 0.447\ P \quad (2)$$

$$Y_{DCP} = 1.44 \times 10^{-7} + 0.972\ P \quad (3)$$

where, P is the P intake. The intercept value of (1.44×10^{-7}) is negligible and in practical use can be omitted from the equations.

Estimation of phosphorus relative bioavailability (RBV)

From equations [2] and [3], the relative bioavailability value for bacterial P is:

$$RBV = bBacteria/bDCP = 0.447/0.972 = 0.46 \quad (4)$$

This means that approximately 0.46 g of P from DCP will yield approximately the same amount of digestible phosphorus (corrected for endogenous phosphorus loss) as 1g of P from the bacterial source. In the rat-model Sehested et al. (personal communication) found that digestibility of rumen bacterial P was 43%, which is very close to 46% in the present study.

Finney (1978) discussed computation of the so-called fiducial intervals for relative bioavailability values. Fiducial intervals are used in practice for essentially the same purpose as confidence limits, although they are based on different philosophical foundations. Computation of fiducial intervals requires the covariance matrix of the vector of parameter estimates in Eq. [1 to 3]. Consider a regression model written in standard matrix notation $Y = X\beta + \varepsilon$. If the errors are independently distributed with common variance δ^2, then the covariance matrix of the vector of parameter estimates is $V(\beta) = \delta^2 (\mathbf{X'X})^{-1}$. The matrix $(\mathbf{X'X})^{-1}$ is:

$$(\mathbf{X'X})^{-1} = (10^{-5}) \cdot \begin{bmatrix} 25{,}581 & -277.4243 & -264.3928 & -308.1471 \\ -277.4243 & 4.98619 & 2.86731 & 3.34183 \\ -264.3928 & 2.86731 & 6.06193 & 3.18485 \\ -308.1471 & 3.34183 & 3.18485 & 6.65223 \end{bmatrix}$$

The (estimated) covariance matrix is obtained by multiplying the MSE times $(\mathbf{X'X})^{-1}$. In this manner the variance of the standard source (DCP) regression coefficient is:

$$MSE.(10^{-5})(4.986) = (71.87)(10^{-5})(4.99) = (0.059864)^2 \quad (5)$$

A simplified version of the fiducial limits presented by Finney (1978) can be used when quantity g is small enough to be negligible. The g value is $(t \cdot (\text{SE of } b_{DCP})/(b_{DCP}))^2$, where t is the 0.05 two-sided critical value for a t distribution with degrees of freedom for MSE, and b_{DCP} is the slope of the standard source regression line. Thus, for the DCP source in phosphorus absorbability data:

$$g = ((2.093)(0.059864)/(0.972))^2 = 0.0166 \quad (6)$$

Considering 0.0166 negligible and following the logic of Finney (1978), the variance for the RBV of bacterial phosphorus can be calculated as:

$$V(RBV) = (71.872)(10^{-5})[6.062 - 2(RBV)(2.8673) + (RBV)^2(4.986)]/(0.972)^2 = (71.872)(10^{-5})[6.062 - 2(0.46)(2.8673) + (0.46)^2(4.986)]/(0.972)^2 = 0.003406. \quad (7)$$

Thus, an approximate standard error for RBV of bacterial phosphorus is $(0.003406)^{1/2} = 0.058$. Approximate 95% fiducial limits for RVB are then:

Table 4. Endogenous phosphorus loss and digestibility of different phosphorus sources in the chick-model.

Test source	P digestibility*	SE
Endogenous P loss (mg/day)	8.265	5.510
Rumen protozoa (mg/g)	94.532[a]	6.914
Rumen bacteria (mg/g)	44.660[b]	6.600
Dicalcium phosphate (mg/g)	97.227[a]	5.986

* Corrected for endogenous P loss, SE: Standard error,

[a–b]Means with the same letter are not significantly different (P<0.05).

$$(RBV–2(0.058),RBV+2(0.058))=(0.344,0.576) \quad (8)$$

Therefore, while the RBV of bacterial phosphorus estimate is 0.46, the reader can be 95% confident that the true RBV of bacterial phosphorus is between 0.344 and 0.576.

The low phosphorus bioavailability in bacteria may be explained by this fact that bacteria can store P as polyphosphates (Weltin et al., 1996) and, it has been shown that digestibility of organic polyphosphates is negligible in intestine (Viljoen, 2003; Soares, 1995; Devereux et al., 1994). Phytic acid has been shown to have a strong antinutritive effect (Pallauf and Rimbach, 1996). This effect is based on the unusual molecular structure of phytic acid Literature on phytic acid existence in rumen bacterial cells is scarce, but the report of Smith et al. (1994) is an evidence of phytate nature of rumen bacterial phosphorus. Therefore, based on findings of Smith et al. (1994), a portion of phosphorus in rumen bacteria is in the form of phytate. In this regards, low RBV of rumen bacteria in the present study may partly be related to the phytate entity of rumen bacterial phosphorus.

In same manner, it is possible to calculate RBV of protozoal phosphorus with confidence interval as follows:

$$RBV = b_{Protozoa}/b_{DCP} = 0.945/0.972= 0.972 \quad (9)$$

It means, approximately 0.972 g of P from the DCP source will yield approximately the same amount of digestible phosphorus (corrected for endogenous phosphorus loss) as 1g of P from protozoa.

$$V(RBV) = (71.872)\ (10^{-5})\ [6.652 – 2\ (RBV)\ (3.3418) + (RBV)^2\ (4.986)]/(0.972)^2$$
$$= (71.872)\ (10^{-5})\ [6.652 – 2\ (0.972)\ (3.34183) + (0.972)^2\ (4.986)]/(0.972)^2$$
$$= 0.0037007. \quad (10)$$

Thus, an approximate standard error for RBV of protozoa phosphorus is $(0.0037007)^{1/2} = 0.061$. Approximate 95% fiducial limits for RVB are then:

$$(RBV–2(0.061), RBV+2(0.061)) = (0.85, 1.094) \quad (11)$$

No reports have been published on P bioavailability of rumen protozoa. Since "availability" of phosphorus is a relative term and is dependent on the standard reference sources used, it is possible to obtain results that are greater than 1. The result of estimates indicated that in some cases protozoal phosphorus bioavailability will be higher than P availability from DCP (1.094 vs. 1.00). In this regards, it is possible to evaluate other microbial phosphorus bioavailability such as bacteria or fungi relative to rumen protozoa as a reference. In the the present study, RBV of phosphorus from rumen bacteria relative to phosphorus from rumen protozoa can be calculated as:

$$RBV \text{ (bacteria relative to protozoa)} = b_{Bacteria}/b_{Protozoa}=0.447/0.945= 0.473 \quad (12)$$

It means, approximately, 0.473g of P from the protozoa will yield approximately the same amount of digestible phosphorus (corrected for endogenous phosphorus loss) as 1g of P from the bacterial source. The variance of the standard source regression coefficient is:

$$MSE.(10^{-5})\ (6.65223) = (71.87164)\ (10^{-5})\ (6.65223) = (0.06914526)^2 \quad (13)$$

The RBV variance can be calculated as:

$$V(RBV) = (71.872)\ (10^{-5})\ [3.18485– 2\ (RBV)\ (6.06193) + (RBV)^2\ (6.65223)\ ]/(0.94531)^2$$
$$= (71.872)\ (10^{-5})\ [3.18485– 2\ (0.473)\ (6.06193) + (0.473)^2\ (6.65223)]/(0.94531)^2$$
$$= 0.000482. \quad (14)$$

Thus, an approximate standard error for RBV is $(0.000482)^{1/2} = 0.022$.

Approximate 95% fiducial limits for RVB are then:

$$(RBV–2(0.022),RBV+2(0.022))=(0.451,0.495) \quad (15)$$

Unlike bacteria, protozoa do not have cell walls. The indigestible portion of microbes is the cell wall, which in bacteria is composed of peptidoglycans. On the other hands, the digestibility of protozoa is higher than bacteria, because larger organisms with less surface to total mass are on the average more digestible (Van Soest, 1994). In particular, the protozoal nutrient components, although of similar biological value to bacterial nutrient components, are readily digested (McNaught et al., 1954; Bergen et al., 1968).

Implications

The results of this study indicated that in formulating diets for dairy cattle, contribution of rumen bacterial and protozoal phosphorus should be taken into account. If the weight of the rumen contents is approximately 100 kg in an adult cow, it is estimated that approximately 4 kg of total microbial biomass is present in this compartment, half of which (2 kg) is represented by the protozoa pool and the other half (2 kg) is represented by bacteria (Jean-Pierre, 2005). Total bacterial and protozoal biomass contains about 87.1 g P (27.7 g bacterial P + 59.4 g protozoal P), providing 50.82 g available P amounting to nearly 76% of phosphorus requirements and almost 24% of that provided directly by forages, concentrates and mineral supplements.

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