The effect of dietary calcium inclusion on broiler gastrointestinal pH: Quantification and method optimization

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ABSTRACT

There is little consensus as to the most appropriate methodology for the measurement of gastrointestinal pH in chickens. An experiment was conducted to establish the optimum sampling method for the determination of broiler digesta pH in birds fed differing levels of dietary calcium. Ross 308 broilers (n = 60) were fed one of two experimental diets, one containing 0.8% monocalcium phosphate and 2% limestone and one containing 0.4% monocalcium phosphate and 1% limestone. Four factors were investigated to determine the most appropriate method of measuring broiler gastrointestinal digesta pH: removal from the tract, prolonged air exposure, altering the temperature of the assay, and controlling the water content of the digesta. The conditions were assessed at bird ages from 7 to 42 d post hatch. Dietary Ca content had no significant effect on in situ pH, but it contributed towards variance in ex situ pH of both gizzard and duodenum digesta. Digesta pH read to be higher when the digesta was removed from the tract, but the amount of time the digesta was exposed to air did not affect the reading. Digesta pH read higher when measured at room temperature than when measured at 41°C; temperature made the strongest unique contribution to explaining variance in duodenum pH, and the second strongest contribution to explaining variance in gizzard pH, after diet. When water was added to the digesta, prior to pH determination, the pH of the digesta read higher ($P < 0.001$) than when measured in situ. The method that resulted in pH readings that were most representative of bird gastrointestinal environment was insertion of a pH probe directly into the gut lumen post euthanasia, because measurement ex situ likely encourages dissociation of carbonic acid, the major buffer in the gastrointestinal tract, which causes pH to read to be higher than when measured in situ. This study shows that the method of pH measurement needs careful consideration to ensure the validity of the result.

Key words: broiler, dietary calcium, methodology, pH

INTRODUCTION

Digesta pH is one of the major gastrointestinal (GI) factors which influence nutrient bioavailability (Pang and Applegate, 2007) and the intestinal microbiota (Hajati and Rezaei, 2010). It is imperative that broiler GI pH is kept at a constant optimal level as small changes outside the normal pH ranges (gizzard 1.2-4 and duodenum 5.7-6.5) (Jiménez-Moreno et al. 2009; Pang and Applegate, 2007; Walk et al. 2012) can have significant negative implications on digestion and mineral absorption (Bristol, 2003). Accurate determination of digesta pH in broiler chickens could therefore act as a tool to indicate the potential for optimum for gut health and maximum nutrient absorption.

The current methodologies used for digesta pH determination in broilers are based predominantly on historic techniques, with the most frequently cited being almost thirty years old (Hurwitz, 1980; Clunies and Leeson, 1984). The majority of methods involve the use of a pH meter with a hand held probe, but sample handling prior to pH testing varies among studies; in particular whether the measurement is determined in situ or ex situ. To investigate limestone and phytase effects on intestinal pH, measurements were taken directly from the digesta contents in the lumen by Walk et al. (2012). In this study, a pH probe (Sensorex S175CD, California, USA) was inserted directly into the gut lumen, through openings made by separating the sections of GI tract, immediately post-euthanasia. In this study gizzard pH ranged from 1.76-2.63 and duodenum pH ranged from 5.86-6.24. A similar in situ method was carried out by Zou et al. (2009), based on the method of Manzanilla (2006), to explore the effects of sodium butyrate in the GI tract in which a unipolar electrode (no further details specified) was inserted through small incisions made in the gut wall. The gizzard pH in that study ranged from 3.02–3.21 and duodenum pH ranged from 6.16-6.20. Winget et al. (1962) however measured GI pH in vivo to investigate the effect of fasting on GI pH in laying hens. To acquire small intestine pH, a pH electrode (Radiometer, GK2021) was inserted into an

incision made in the small intestine under anaesthetic, and to obtain gizzard pH the bird swallowed a pH electrode (Radiometer, G282A), and it was forced through the oesophagus into the gizzard. Radiographs were taken to ensure the probes were in the correct position. In this study, gastric pH ranged from 3.17-3.48 duodenal pH ranged from 5.77-7.10. Whilst this method minimises alteration of the gastrointestinal environment through air exposure, its invasiveness precludes general use.

In contrast to the in situ methods discussed above, González-Alvarado et al. (2008), Jiménez-Moreno et al. (2009) and Engberg et al. (2004) removed the digesta prior to measuring pH to explore the effects of fiber source and heat processing, and the effects of whole wheat and xylanase, on GI pH. Gizzard pH ranged from 3.14-3.56 and duodenum pH ranged from 5.72-5.93 in these studies. These findings indicate that gizzard pH tends to be higher when measured ex situ than in situ. This suggests that the impact of removing the digesta from the tract is potentially a key factor affecting GI pH determination. This may be because exposure to air causes carbonate from dietary limestone, blood buffering capacity and pancreatic secretions to dissociate to CO_2 and water (Guinotte et al., 1995), thus resulting in removal of hydrogen ions from the milieu (Zhang and Coon, 1997).

Some of the methods presently used to determine poultry GI pH involve addition of water to the digesta prior to pH determination. For example, to investigate the effect of copper on the GI environment digesta was removed and nine-fold dilution of deionized water was added, based on the digesta weight prior to pH determination (Pang and Applegate, 2007). In this study, pH in the gizzard ranged from 3.07-3.28 and in the duodenum, from 6.22-6.31. The same method was carried out by Houshmand et al. (2011) and Esmaeilipour et al. (2011) to investigate the effect of non-antibiotic feed additives and the effect of xylanase and citric acid, respectively, on the GI environment; gizzard and duodenum pH ranged from 2.85-4.22 and 5.92-6.26 respectively in these studies. To examine the effects of dietary Ca

and fat on intestinal pH Shafey et al. (1991) flushed the GI tract from the base of the gizzard with 2ml distilled water, and then added an additional 5ml to the digesta before measuring pH; duodenal pH in this study ranged from 5.86-6.24. Also, to investigate the effect of citric acid and phytase on GI pH, Nourmohammadi et al. (2011) added 90ml of sterilized physiological saline (1:10 dilution) to 10 g of digesta content; gizzard pH ranged 3.09-3.23 and duodenum 5.71-5.80. Methods involving diluting digesta samples prior to pH determination have been observed as far back as 1969, when Bowen and Waldroup (1969) examined the influence of propylene glycol on GI pH. In that study, the gizzard pH ranged from 2.47-3.06, and the duodenum pH ranged from 5.46-6.65. It can be noted from these results that pH generally reads higher in diluted digesta samples than those determined in situ. This indicates that a further potential issue to consider is variation between samples based on hydrogen ion concentration, that is, how diluted the digesta is by recent water consumption or by addition of water to digesta prior to pH determination.

In laying hens, the impact of varying volume and source of limestone in a diet has been extensively researched, but in broilers there are limited published data. There is a perception that there are no issues surrounding over-inclusion of limestone in broiler diets. However, a combination of both the high buffering capacity of carbonate and an elevated pH caused by presence of Ca leads to raised digesta pH levels (Ekmay and Coon, 2010). An increase in GI pH in broilers fed high Ca from limestone reduced apparent ileal crude protein digestibility (Walk et al., 2012). Although mineral research tends to prioritize P, as it is non-renewable and hence increasingly expensive, the potential negative effects of incorrect limestone supplementation, especially with regards to GI pH, should not be discounted.

The aims of this study were to establish the optimum sampling method for the determination of broiler digesta pH that is most representative of the GI environment and, subsequently, to determine the effect of dietary limestone inclusion level on digesta pH. The

sampling methods assessed were the effect on pH of removing the digesta from the gut, subjecting the digesta to prolonged air exposure, altering temperature of the digesta pH assay, and controlling the amount of water present in the digesta; in birds fed one of two dietary limestone levels.

Birds and Husbandry

Ross 308, male broilers (n = 60) from a 42-week-old breeder flock were obtained from a commercial hatchery at day of hatch. Chicks were randomized by weight and placed in 0.64 m^2 floor pens in groups of six, bedded on clean wood shavings. Birds were allowed ad libitum access to the treatment diets and water for the duration of the trial. The room was thermostatically controlled to produce an initial temperature of 32°C, reduced to 21°C by day 21. The lighting regimen used was 24 hours light on d 1, with darkness increasing by 1 hour a day until 6 hours of darkness was reached, and this was maintained throughout the remainder of the study. All birds sampled were euthanized by cervical dislocation. This occurred at the same time each sampling day; after at least 6 hours of light, to ensure maximal gut fill. Institutional and national guidelines for the care and use of animals were followed, and all experimental procedures involving animals were approved by the University College of Science ethical review committee.

Dietary Treatments

Experimental diets were formulated to be as nutritionally similar as possible, with the exceptions of P and Ca, and to meet the requirements of the age and strain of bird. The low diet was formulated at a low level of Ca and P (0.4% monocalcium phosphate and 1% limestone), and the high diet was formulated to contain double the inclusion levels of Ca and P (0.8% monocalcium phosphate and 2% limestone). These levels were chosen to produce a measurable difference in digesta buffering. This resulted in two dietary treatment groups with each treatment replicated by 5 pens of 6 chicks each (30 chicks/dietary treatment). After

dietary treatment allocation, individual birds within pens were subsequently assigned to a designated sampling method as detailed in the methodology below for each experiment.

The limestone in the diets had a particle size of 1-2mm (average U.S. standard screen number 14). Sodium bicarbonate was added to the diets to reduce total chloride content. Diets were fed in mash form, mixed in house, and were analysed for gross energy by bomb calorimetry (Robins and Firman, 2006), dry matter and protein content (calculated as nitrogen multiplied by 6.25) by the AOAC standard methods (930.15 and 990.03, respectively). Phosphorus and Ca content of the diets were analysed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) following an aqua regia digestion step (AOAC 985.01). Calculated and analysed values for each diet are shown in Table 1.

Acid binding capacity (ABC) and buffering capacity (BUF) of the diets were determined based on the assay of Lawlor et al. (2005). A 0.5 g sample of diet was suspended in 50 ml ultra-pure water with continuous stirring. The suspension was then titrated with 0.1 mol/L HCl so that approximately 10 additions of titrant were required to reach pH 2.0. The pH readings after each addition were recorded following equilibration for 3 minutes. Acid-binding capacity was calculated as the amount of acid in milliequivalents (meq) required to lower the pH of 1 kg food to pH 2, 3 and 4. This was repeated 5 times per diet. The analysed values are presented in Table 2.

Experiment 1: Effect of diet and age on gizzard and duodenum digesta pH in situ

Forty-eight birds were used to assess the effect of varying dietary limestone content and the effect of bird age on digesta pH. Sampling was carried out on 8 birds per day (4 birds on each diet per day), on d 7, 14, 21, 28, 35 and 42 post-hatch. Immediately post euthanasia, the gizzard was removed intact and a digital pH meter (Mettler-Toledo, UK) with a spear tip piercing pH electrode (Sensorex S175CD, California, USA) was directly inserted into the digesta in the lumen of the proximal gizzard (proventricular opening), whilst ensuring the pH

electrode did not touch the gizzard wall, and the pH was recorded. This was repeated six times, putting the probe in different areas of the gizzard each time (mean variability +/- 0.07 SEM). The probe was rinsed with ultra-pure water once all six readings had been taken. The process was then repeated in the duodenal loop of the same bird. Readings were taken at the distal end of the duodenum; based on average length of the duodenum across the bird ages, the duodenum was cut at a point 30 cm from the gizzard (Yadav et al. 2010), and the pH electrode was inserted directly into this opening. Again, measurements were repeated six times (mean variability +/- 0.04 SEM). The tip of the pH probe was stored in pH 4 solution when not in use.

Experiment 2: Effect of removing digesta from the gastrointestinal tract on determining digesta pH

Twenty-four birds were used to assess the effect of removing the digesta from the GI tract on measuring digesta pH. Sampling was carried out on 8 birds per day (4 birds on each diet per day), on d 7, 14 and 28 post-hatch. Immediately post euthanasia, in situ gizzard and duodenal pH were determined, as previously described, for every bird on each sampling day (mean variability +/-0.06 SEM and +/- 0.03 SEM, respectively). For half the birds (n=4, 2 on each diet, per sampling day) the digesta was removed immediately after in situ pH had been determined, and was put into centrifuge tubes that had been maintained at room temperature (14.4°C +/- 0.15 SEM). A stop watch was started the instant the digesta had been put into the centrifuge tubes, and pH was recorded every 15 seconds for three minutes using a spear-tip electrode and digital pH meter. This entire process was carried out on the other half of the birds (n=4, 2 on each diet, per sampling day), except the digesta was put into centrifuge tubes that had been previously warmed to 41°C in a water bath.

Experiment 3: Effect of digesta water content on digesta pH

Thirty-six birds were used to assess the effect of digesta water content on digesta pH. Sampling was carried out on 12 birds per day (6 on each diet per day), on d 21, 35 and 42 post-hatch. Immediately post euthanasia, in situ gizzard pH was determined as previously described (mean variability +/- 0.04 SEM). The digesta contents were then transferred into 7 ml containers and weighed, and then immediately snap frozen using a dry ice/industrial methylated spirit mix. The frozen samples were freeze dried, re-weighed and the average water content across all the samples was calculated. This process was repeated in the duodenum of the same bird (mean variation in situ +/- 0.07 SEM). For each section of the tract, after freeze drying, the samples were reconstituted with a corresponding volume of deionised water (pH 6.95 +/- 0.02 SEM) to ensure uniform water content equal to the average of all samples collected. The pH of the reconstituted digesta samples was then measured directly with six replicate readings per sample for the gizzard and duodenum (mean variability +/- 0.06 and +/- 0.03 SEM, respectively).

Statistical Analysis

All data were analyzed using IBM SPSS statistics version 21. In experiment 1, an ANOVA was conducted to determine 2-way interactions between bird age and dietary limestone content on in situ gizzard and duodenum pH. When means were significantly different, t-tests were conducted to differentiate between means. Statistical power calculations were used to predict sample size that would be required to predict differences in dietary limestone content effect at different pH measures. In experiment 2, multiple linear regressions, with individual bird number as a covariate, were used to determine the unique contribution and relatedness of time exposed to air (log time (seconds)), digesta temperature, and diet on variance in gizzard and duodenal pH at d 7, d 14 and d 28. Interpretations of the strength between the relationships were based on those of Cohen (1988): small r = 0.1-0.29, medium r = 0.30-0.39 and large r = 0.50 to 1.0. T-tests were conducted to make statistical

comparisons between in situ pH and pH at the exponential time point where digesta pH ceased to fluctuate post-removal from the tract. Two-, 3- and 4-way interactions between diet, time exposed to air, digesta temperature, and bird age were determined by multiple ANOVA. In experiment 3, t-tests were conducted to make statistical comparisons between in situ pH and the pH readings of the samples that had been reconstituted with water. Two- and 3-way interactions among diet, bird age, and sampling method (in situ or reconstituted with known water content) were determined by multiple ANOVA. Multiple linear regressions, with individual bird as a covariate, were used to determine the unique contribution and relatedness of digesta water content and diet on variance in gizzard and duodenal pH at d 21, d 35 and d 42. Pearson product-moment correlation coefficient was carried out to investigate the relationship between in situ pH and digesta DM at d 21, d 35 and d 42. Significance was always accepted at $P < 0.05$.

RESULTS AND DISCUSSION

This series of experiments investigated both the effect of dietary Ca level on digesta pH in broilers, and also whether pH is altered by sample retrieval methods. Only significant interactions are presented in the tables and discussed. If the interactions were not significant, the main effects were discussed.

Effect of diet and age on in situ gizzard and duodenum pH

The in situ gizzard and duodenal pH values in this study were not significantly different to those found throughout the literature, such as those presented by both Walk et al. (2012) and Zou et al. (2009). In experiment 1, an interaction ($P < 0.05$) was observed between bird age and dietary limestone content on in situ gizzard pH (Table 3). In general, gizzard pH fluctuated substantially (1.8 to 3.6) among the days measured. This may be partly due to the time that the birds were euthanized prior to sample collection; the anterior tract is emptied during dark periods suggesting that feed intake (May et al. 1990), and thus retention

time in the tract, may vary between birds. Another possible explanation for this variation is that the birds were fed a mash diet and hence may have selected Ca from the diets (Wilkinson et al. 2011) and modified diet consumption based on Ca requirements.

There was no relationship between gizzard pH and bird age, which is in agreement with the work of Angel et al. (2001). Gizzard pH was, however, significantly higher in birds fed the high limestone compared to birds fed the low limestone diet on d 7, 14 and 35 (Table 3). This may be largely due to the greater buffering capacity of the high limestone diets compared to the low limestone diets (Table 2). Similar findings have been observed throughout the literature; for example gizzard pH was 2.37 compared to 2.52 in birds (aged d 0-16) fed either a diet containing 0.64% or 1.03% Ca, respectively, in a study conducted by Walk et al. (2012), and in a study by Guinotte et al. (1995) gizzard pH in immature birds was 2.76 compared to 3.82 in diets containing either 10g/kg or 36g/kg Ca respectively. This observed increase in pH with higher dietary limestone content in the gizzards of generally younger birds may be because they are more vulnerable to alterations in the gastrointestinal environment, and they are unable to react to the increased bicarbonate load by increasing proventricular HCl secretion, due to the immaturity of the gizzard (Coutu and Craig, 1988; Winkler et al., 1996). This however does not explain the re-emergence of this observed finding in the d 35 birds.

Conversely, on d 28 and d 42 gizzard pH was higher in birds fed the low limestone diet, and diet had no influence on gizzard pH at d21. This finding is difficult to reconcile alongside findings from other ages. A possible explanation is that feed intake was increased and gizzard retention time reduced in order to meet the high demand for Ca (Zhang and Coon, 1997), thereby exceeding capacity to secrete sufficient HCl to maintain acidity of digesta in the gizzard. Unfortunately, feed intake, relative gizzard size and digesta transit rate were not measured in this study, so this theory cannot be verified at this point. The low sampling sizes,

and high variability in gizzard pH, suggest that further investigation with more birds is needed to fully evaluate these findings.

In the duodenum no interactions or significant effects of dietary Ca level or bird age were observed on digesta pH (data not shown). Previous studies have suggested that alteration in gizzard pH subsequently impacted duodenal pH via manipulation of bacterial colonisation of the lower digestive tract (Duke, 1992; Fernandez et al., 2002). However, the current study does not reflect this finding. This may be due to methodical differences in the sample handling prior to pH measurement. The number of birds necessary to predict differences in duodenum pH between the two diets was highest at d 21 and lowest at d 35, with 25 birds and 9 birds required, respectively. Similar figures were also observed in the gizzard (Table 3) highlighting that variation between individual birds, regarding the effect of diet on gastrointestinal pH, is detectable in both the gizzard and duodenum. This, however, requires further investigation, as there was slight variation between numbers of birds required at the other bird ages.

Effect of removing digesta from the gastrointestinal tract on determining digesta pH

In experiment 2, there was no effect ($P > 0.05$) of temperature x diet x age on gizzard pH. However, gizzard pH was significantly higher in birds fed the high limestone diet compared to those fed the low limestone diet, but only on d 14. There was no effect of diet on gizzard pH on d 7 or d 28, but there was a numerical increase in gizzard pH in birds fed the high limestone diet at d 7 which resulted in a diet x age interaction ($P < 0.05$; Table 4). This increase in pH caused by high dietary limestone presence has possible negative implications for Ca and P utilization, because at high pH hydrolysis of phytate-Ca complexes are reduced, as most microbial phytases are active only at low pH. Additionally, at low pH Ca and P are relatively soluble, and are hence unlikely to precipitate, but at higher pH

289 phytate-mineral complexes are more insoluble (Selle et al., 2000), so precipitation of Ca, P
290 and phytate is likely. Gizzard pH decreased from d7 to d28 which may be due to an increase
291 in dry matter content of the digesta due to heightened feed intake. The findings from this
292 study suggest that high dietary inclusion levels of limestone potentially has a detrimental
293 effect on gut pH, but further investigation using a larger population of broilers would be
294 needed to fully identify the extent of this effect on phytate.

295 Maintaining samples at room temperature after removal from the tract led to gizzard
296 pH readings being consistently higher ex situ than in situ, but when the digesta pH was
297 measured ex situ in samples maintained at 41°C, this was not always the case (Table 4). An
298 interaction ($P < 0.05$) was observed between temperature and bird age on digesta pH in the
299 gizzard (Table 4). On d 7 and 28, gizzard pH was significantly higher when measured at
300 room temperature than when measured at 41°C, but temperature had no effect on gizzard
301 digesta pH on d 14. Similar to the gizzard, duodenum pH was numerically higher when
302 measured at room temperature than when measured at 41°C, with the exception of d 28 in
303 birds fed the high diet, where duodenum pH was the lowest and not affected by temperature x
304 age ($P < 0.05$; Table 4). This may be due to the small sampling size, gut maturity or high
305 variability in duodenum pH. Digesta temperature made the strongest unique contribution to
306 duodenum pH, and second strongest contribution to gizzard pH, when the effects of diet and
307 time exposed to air were controlled for, and digesta temperature and pH were correlated
308 (Table 5). The observed findings may have been confounded by individual bird variation,
309 thus further investigation is needed to fully consider the interaction between digesta
310 temperature and bird age. This again highlights that measuring digesta pH in situ is likely to
311 provide pH readings that are most representative of the GIT environment of the bird.

312 The time of digesta exposure to air had no significant effect on gizzard or duodenum
313 digesta pH, but initial removal of digesta from the tract lead to a numerical rise in pH before

the readings plateaued (data not shown). This plateau may indicate the point at which no further CO_2 remains to be released from the carbonate in the digesta. Although time exposed to air had no significant effect on digesta pH, it did make the biggest unique contribution towards the variance observed in duodenal pH in 14 d-old birds (Table 5). This may be because at this bird age there were more Ca ions present in the digesta to influence pH. The effect of time exposure did not, however, significantly affect duodenal pH at this bird age because the factors of diet, time exposed to air and digesta temperature accounted for only 24% of the variance in duodenal pH (Table 5). The generally observed increase in pH when measured ex situ compared to in situ in both the gizzard and duodenum (Table 4) is potentially attributable to CO_2 release from carbonate buffering pH on exposure to air by altering the equilibrium of carbonic acid dissociation towards water and CO_2. Further investigation is required to confirm this. It can therefore be speculated that a combination of both heightened pH buffering effect and reduced digesta temperature on exposure to air contributed to the observed increase in pH on removal of digesta from the tract. This suggests that measuring pH of digesta that has been removed from the tract may not be providing a true representation of any dietary effects on the GIT environment.

Effect of digesta water content on digesta pH

In experiment 3, digesta from both the gizzard and duodenum were standardised with a known volume of water to identify the effect of dilution on the acidity of the sample. This was investigated to identify the influence of variation in water consumption by the bird on digesta pH. A secondary aim of this study was to identify if water addition to the sample prior to pH determination, as observed in published studies such as Pang and Applegate (2007), Smulikowska et al. (2009) and Mirzaie et al. (2012), was impacting on the accuracy of the pH reading.

Diet had no effect on gizzard or duodenal pH in experiment 3 (data not shown). Digesta pH read higher ($P < 0.05$) in the samples that had been reconstituted with water compared to the in situ measurements in the gizzard and the duodenum (Table 6). The addition of water dilutes hydrogen ions thereby reducing the acidity of the digesta. Despite both studies using the same range in dietary Ca concentration between treatments, Shafey et al. (1999) found a significant effect of dietary Ca on digesta pH which was not found in this study. This may be due to the substantial amount of distilled water (approximately 7ml) added to the digesta prior to pH measurement in the study conducted by Shafey et al. (1999). In the current study, the observed higher pH in the reconstituted samples suggests that adding water to digesta, coupled with removing the digesta from the tract before reading the pH, potentially reduces the accuracy of the reading and does not necessarily reflect the GIT environment within the bird. Further investigation is needed into the influence that variation in water consumption may have on digesta pH, as the method used in this study observes only the impact of a singular level of reconstitution on digesta pH.

It is likely that freeze-drying had little direct effect on the pH of the digesta, or influence on the higher pH observed in the reconstituted samples (Table 6). This is based on the general acceptance that chemical reactivity in solid form corresponds to the pH of the aqueous solution prior to freeze-drying; referred to as 'pH memory' (Govindarajan et al. 2006). Numerous studies observing the impact of freeze-drying on sample pH, for example Costantino et al. (1997) and Vakos et al. (2000), found that pH and behaviour of proteins in aqueous states were similar to those presented in the same solution post freeze-drying.

Digesta dry matter content of both the gizzard and jejunum was numerically higher in birds fed the low limestone diet compared to those fed the high limestone diet over bird age d 21, 35 and 42 (gizzard 502.50g/kg +/- 12.98 SEM and 467.74g/kg +/-19.23 SEM, respectively, and duodenum 396.37g/kg +/- 12.49 SEM and 393.71g/kg +/- 14.77 SEM,

363 respectively). This may be because feed intake of the low limestone diet was higher, to meet
364 the demands for Ca. There were strong correlations between digesta DM and in situ pH in the
365 gizzard at d21 (r= -0.765), d35 (r= -0.649) and d42 (r= -0.682), and in the duodenum at d21
366 (r= 0.550), d35 (r= 0.720) and d 42 (r= -0.741), where confidence in the result was always
367 $P<0.05$. This supports the supposition that digesta water content influences GIT pH. This is
368 also illustrated in Table 7, whereby reconstitution with water was shown to make the biggest
369 unique contribution towards the variance observed in duodenal pH, when the contribution of
370 diet was accounted for, at all the bird ages in this experiment. Reconstitution with water also
371 made the biggest unique contribution to gizzard at d 42, and made relatively high
372 contributions in the other bird ages in this experiment. As bird age increased impact of water
373 content and diet on the variance in pH in both the gizzard and duodenum decreased (Table 7),
374 likely due to increased gut maturity and hence ability to respond to alterations to the GI
375 environment.

376 Sample handling profoundly affects pH determination in digesta. A key factor seems
377 to be removal of the digesta sample from the tract, as this appears to cause pH to alter from
378 the in situ value. Removal of digesta from the bird also affects pH via an associated
379 temperature reduction, which can be partially mitigated through use of a water bath to
380 maintain bird body temperature. However, this approach is not recommended as the buffering
381 effect upon removal cannot be overcome. Water content of the digesta was also shown to
382 have a substantial effect on pH, but this could not be standardised without confounding
383 results by removing the digesta from the tract. It can be concluded that the method that gives
384 the most accurate representation of broiler GIT environment when determining digesta pH is
385 to insert a pH probe directly in situ into the gut lumen immediately post euthanasia. Generally,
386 pH was higher in birds fed the high limestone diet compared to birds fed the low limestone
387 diet, suggesting that excessive dietary limestone levels in broiler diets potentially has

388 negative implications on GIT pH. However, this conclusion requires verification in a larger
389 study using the optimum sampling techniques described above and a wider range of
390 limestone levels.

391 **REFERENCES**

392 Angel, R., B. Humphreys and W. Saylor. 2010. Age changes in gastrointestinal pH in broilers.
393 Poult. Sci. 89, E-Suppl. 1.
394
395 Borgström, B. 1982. The temperature-dependent interfacial inactivation of porcine pancreatic
396 lipase. Effect of colipase and bile salts. Biochimica. Biophysica. acta. 712:490-497.

397 Bowen, T.E. and Waldroup, P.W. 1969. The influence of propylene glycol on pH of the
398 gastrointestinal tract and the incidence of leg abnormalities in broiler chicks. Poult. Sci.
399 48: 608-613.

400 Clunies, M. and S. Leeson. 1984. In vitro estimates of dry matter and crude protein
401 digestibility. Poult. Sci. 63:89-96.

402 Cohen, J. 1988. Statistical power analysis for the behavioural sciences. 2nd ed. Hillsdale, NJ,
403 Erlbaum.

404 Costantino, H.R., K. Griebenow, R. Langer and A.M. Klibanov. 1997. On the pH memory of
405 lyophilized compounds containing protein functional groups. Biotechnol. Bioeng., 53:
406 345–348.

407 Coutu, M. and S. W. Craig. 1988. cDNA-derived sequence of chicken embryo vinculin. Proc.
408 Natl. Acad. Sci. 85:8535-8539.

409 Duke, G.E. 1992. Recent studies on regulation of gastric motility in turkeys. Poult. Sci. 71:1-
410 8.

411 Ekmay, R.D. and C. N. Coon. 2010. The effect of limestone particle size on the performance
412 of three broiler breeder purelines. Int. J. Poult. Sci. 9:1038-1042.

413 Engberg RM, M.S. Hedemann, S. Steenfeldt and B.B. Jensen. 2004. Influence of whole
414 wheat and xylanase on broiler performance and microbial composition and activity in the
415 digestive tract. Poult. Sci. 83: 925-938.

416 Esmaeilipour O, M. Shivazad, H. Moravej, S. Aminzadeh, M. Rezaian, M.M. van Krimpen.
417 2011. Effects of xylanase and citric acid on the performance, nutrient retention, and
418 characteristics of gastrointestinal tract of broilers fed low-phosphorus wheat-based diets.
419 Poult. Sci. 90: 1975-1982.

420 Fernandez, F., M. Hinton, and B. Van Gils. 2002. Dietary mannan-oligosaccharides and their
421 effect on chicken caecal microflora in relation to Salmonella enteritidis colonization.
422 Avian Pathol. 31:49-58.

Geyra, A., Z. Uni and D.Sklan. 2001a. Enterocyte dynamics and mucosal development in the post-hatch chick. Poult. Sci. 80:776-782.

Geyra, A., Z. Uni and D.Sklan. 2001b. Effect of fasting at different ages on growth and tissue dynamics in the small intestine of the young chick. Br. J. Nutr. 86:53-61.

González-Alvarado JM, E. Jiménez-Moreno, D.G. Valencia, R. Lázaro and G.G. Mateos. 2008. Effects of fiber source and heat processing of the cereal on the development and pH of the gastrointestinal tract of broilers fed diets based on corn or rice. Poult. Sci. 87: 1779-1795.

Govindarajan, R., K. Chatterjee1, L.Gatlin, R. Suryanarayanan, E. Y. Shalaev. 2006. Impact of freeze-drying on ionization of sulfonephthalein probe molecules in trehalose–citrate systems. J. Pharm. Sci. 95: 1498–1510.

Guinotte, F., J. Gautron, Y. Nys, and A. Soumarmon. 1995. Calcium solubilization and retention in the gastrointestinal tract in chicks (Gallus domesticus) as a function of gastric acid secretion inhibition and of calcium carbonate particle size. Br. J. Nutr. 73:125-139.

Guo, W., J. L. Mosenfelder, W. A. Goddard, and J. M. Eiler. 2009. Isotopic fractionations associated with phosphoric acid digestion of carbonate minerals: Insights from first-principles theoretical modeling and clumped isotope measurements. Geochimica. Cosmochimica. Acta. 73:7203–7225.

Hajati, H. and M. Rezaei. 2010. The application of prebiotics in poultry production. Int. J. Poult. Sci. 9:298-304.

Han, B. K., J. K. Moon, Y. W. Ryu, Y. -H. Park, and D. -H. Jo. 2000. Purification and characterisation of acidic chitinases from gizzards of broiler. J. Biochem. Mol. Bio. 33:326-331.

Houshmand, M., K. Azhar, I. Zulkifli, M. H. Bejo, A. Meimandipour and A. Kamyab. 2011. Effects of non-antibiotic feed additives on performance, tibial dyschonroplasia incidence and tibia characteristics of broilers fed low calcium diets. J. Anim. Physiol. Anim. Nutr. 95: 351-358

Hume, M. E., L. F. Kubena, T. S. Edrungton, C. J. Donskey, R. W. Moore, S. C. Ricker, and D. J. Nisbet. 2003. Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. Poult. Sci. 82:1100-1107.

Hurwitz, W. 1980. Official methods of analysis. Asso. Anal. Chem. 13th ed. Washington D.C., USA.

Jiménez-Moreno, E., J. M. González-Alvarado, R. Lázaro and G. G. Mateos. 2009. Effects of type of cereal, heat processing of the cereal, and fiber inclusion in the diet on gizzard pH and nutrient utilization in broilers at different ages. Poult. Sci. 88: 1925-1933.

Jin, S..H., A. Corless and J. L. Sell. 1998. Digestive system development in post-hatch poultry. World's Poult. Sci. J. 54:335-345.

461 Lawlor, P.G., P. B. Lynch, P.J. Caffrey, J.J. O'Reilly and M.K. O'Connell. 2005.
462 Measurements of the acid-binding capacity of ingredients used in pig diets. Ir Vet J.
463 58(8): 447–452.

464 Marchini, C. F. P., P. L. Silva, M. R. B. M. Nascimento, M. E. Beletti, N. M. Silva, and E. C.
465 Guimaraes. 2011. Body weight, intestinal morphometry and cell proliferation of broiler
466 chickens submitted to cyclic heat stress. Int. J. Poult. Sci. 10:455-460.

467 Martinez-Haro, M., M. Taggart, A. J. Green, and R. Mateo. 2009. Avian digestive tract
468 simulation to study the effect of grit geochemistry and food on Pb shot bioaccessibility.
469 Environ. Sci. Tech. 43:9480-9486.

470 Manzanilla, E. G. , J. F. Pérez, M. Martín, J. C. Blandón, F. Baucells, C. Kamel and J. Gasa.
471 2009. Dietary protein modifies effect of plant extracts in the intestinal ecosystem of the
472 pig at weaning. J. Anim. Sci. 87:2029-2037.

473 Manzanilla, E. G., M. Nofrarias, M. Anguita, M. Castillo, J. F. Perez, S. M. Martin-Orue, C.
474 Kamel, and J. Gasa. 2006. Effects of butyrate, avilamycin, and a plant extract
475 combination on the intestinal equilibrium of early-weaned pigs. J. Anim. Sci. 84:2743–
476 2751.

477 May, J.D., B.D. Lott and J.W. Deaton. 1990. The effect of light and environmental
478 temperature on broiler digestive tract contents after feed withdrawal. Poult. Sci. 69:
479 1681-1684.

480 Mirzaie, S., M. Zaghari, S. Aminzadeh, and M. Shivazad. 2012. The effects of non-starch
481 polysaccharides content of wheat and xylanase supplementation on the intestinal
482 amylase, aminopeptidase and lipase activities, ileal viscosity and fat digestibility in layer
483 diet. Iran J. Biotech. 10:208-214.

484 Nitsan, Z., E. A. Dunnington, and P. B. Siegel. 1991. Organ growth and digestive enzyme
485 levels to fifteen days of age in lines of chickens differing in body weight. Poult. Sci.
486 70:2040-2048.

487 Nourmohammadi, R., S. M. Hosseini, H. Saraee, A. Arab, and H. Arefinia. 2001. Plasma
488 thyroid hormone concentrations and pH value of some GI-tract segments of broilers fed
489 on different dietary citric acid and microbial phytase levels. J. Anim. Vet. Adv. 10:1450-
490 1454.

491 Noy, Y. and D. Sklan. 1995. Digestion and absorption in the young chick. Poult. Sci. 74:366-
492 376.

493 Pang. Y. and T. J. Applegate. 2007. Effects of dietary copper supplementation and copper
494 source on digesta pH, calcium, zinc, and copper complex size in the gastrointestinal tract
495 of the broiler chicken. Poult. Sci. 86:531-537.

496 Revolledo, L., A. J. P. Ferreira, and G. C. Mead. 2006. Prospects in salmonella control:
497 Competitive exclusion, probiotics, and enhancement of avian intestinal immunity. J.
498 Appl. Poult. Res. 15:341-351.

499 Robbins, D. H. and J. D. Firman. 2006. Evaluation of the metabolizable energy of poultry by-
500 product meal for chickens and turkeys by various methods. J. Poult. Sci. 5(8): 753-758.

501 Sell. J. L. 1996. Physiological limitation and potential improvement in gastrointestinal tract
502 function of poultry. J. Appl. Poult. Res. 5:96-101.

503 Selle, P. H., A. J. Cowieson, and V. Ravindran. 2009. Consequences of calcium interactions
504 with phytate and phytase for poultry and pigs. Livest. Sci. 124:126-141.

505 Selle, P. H., V. Ravindran, R. A. Caldwell, and W. L. Bryden. 2000. Phytate and phytase:
506 Consequences for protein utilisation. Nutr. Res. Rev. 13:255–278.

507 Shafey, T. M. 1999. Effects of high dietary calcium and fat levels on the performance,
508 intestinal pH, body composition and size and weight of organs in growing chickens. Am.
509 J. Anim. Sci. 12:49-55.

510 Shafey. T. M., M. W. McDonald, and J. G. Dingle. 1991. Effects of dietary calcium and
511 available phosphorus concentration on digesta pH and on the availability of calcium,
512 iron, magnesium and zinc from the intestinal contents of meat chickens. Br. J. Poult. Sci.
513 32:185-194.

514 Sklan, D. 2001. Development of the digestive tract of poultry. World's Poult. Sci. J. 57:415-
515 428.

516 Smith. D. P. and M. E. Berrang. 2006. Prevalence and numbers of bacteria in broiler crop and
517 gizzard content. Poult. Sci. 85:144-147.

518 Smulikowska, S., J. Czerwiński, A. Mieczkowska, and J. Jankowiak. 2009. The effect of fat-
519 coated organic acid salts and a feed enzyme on growth performance, nutrient utilization,
520 microflora activity, and morphology of the small intestine in broiler chickens. J. Anim.
521 Feed. Sci.18:478–489.

522 Tanaka, A. and E. Hoshino. 2003. Secondary calcium-binding parameter of Bacillus
523 amyloliquefaciens alpha-amylase obtained from inhibition kinetics. J. Biosci. Bioeng.
524 96:262-267.

525 Tanid, I. 1992. Effect of early postnatal long-term fasting on the development of peptide
526 hydrolysis in chicks. Comp. Biochem. Physiol. 101:161-166.

527 Vaintraub. I. A. and V. P. Bulmaga. 1991. Effect of phytate on the in vitro activity of
528 digestive proteinases. J. Agric. Food. Chem. 39:859-861.

529 Vakos H.T., H. Kaplan, B. Black , B. Dawson and M.A. Hefford. 2000. Use of the pH
530 memory effect in lyophilized proteins to achieve preferential methylation of alpha-amino
531 groups. J. Protein Chem. 19: 231-7.

532 Volberg, T., H. Sabanay, and B. Geigra. 1986. Spatial and temporal relationships between
533 vinculin and talin in the developing chicken gizzard smooth muscle. Differentiation.
534 32:34-43.

535 Walk, C. L., M. R. Bedford, and A. P. McElroy. 2012. Influence of limestone and phytase on
536 broiler performance, gastrointestinal pH, and apparent ileal nutrient digestibility. Poult.
537 Sci. 91:1371-1378.

538 Wilkinson, S.J., P.H. Selle, M.R. Bedford and A.J. Cowieson. 2012. Exploiting calcium-
539 specific appetite in poultry nutrition. World's Poult. Sci. 67:587-598.

540 Winget, C.M., G.C. Ashtn and A.J. Cawley. 1962. Changes in gastrointestinal pH associated
541 with fasting in the laying hen. Poult. Sci. 41: 1115-1120.Winkler, J., H. Lünsdorf, and B.
542 M. Jockusch. 1996. The ultrastructure of chicken gizzard vinculin as visualized by high-
543 resolution electron microscopy. J. Struct. Biol. 116:270-277.

544 Yadav, G. B., A.S. Kadam, A.M. Pachpande, S.B. Lamabate, V.D. Lonkar, S. Maini and K.
545 Ravikanth. 2010. Post hatch histo-morphological studies of small intestinal development
546 in chicks fed with herbal early chick nutritional supplement. Int. J. Poult. Sci. 9:851-855.
547
548 Zhang, B. and C. N. Coon. 1997. The relationship of calcium intake, source, size, solubility
549 in vitro and in situ, and gizzard limestone retention in laying hens. Poult. Sci. 76:1702-
550 1706.
551 Zou, Y., Z. B. Yang, W. R. Yang, S. Z. Jiang, G. G. Zhang, R. Yu. 2009. Effects of sodium
552 butyrate on gut pH value, gut VFA and cecal microflora of broilers.
553 http://www.kdqfeed.com/English/admsys/pdf/upfile/201131011292757163.pdf.
554 Accessed March 2013.
555

Table 1. Composition and nutrient content of experimental diets

Ingredient	High diet (%)	Low diet (%)
Wheat	66.4	67.8
Soybean meal, 48% CP	25.0	25.0
Lysine	0.30	0.30
Methionine	0.25	0.25
Soy oil	4.00	4.00
Limestone	2.00	1.00
Monocalcium phosphate	0.80	0.40
Sodium chloride	0.25	0.25
Sodium bicarbonate	0.15	0.15
Broiler Trial Supplement[1]	0.40	0.40
TiO_2	0.50	0.50
Calculated composition		
Crude protein, %	20.1	20.3
GE, kcal/kg	4660	4660
Total P, %	0.55	0.46
Total Ca, %	1.10	0.65
Lys, %	1.23	1.24
Met, %	0.52	0.53
Total sulphur amino acids %	0.84	0.84
Sodium, %	0.18	0.18
Potass, %	0.80	0.80
Chloride, %	0.27	0.27
Analysed composition		
Crude protein, %	20.8	20.4
GE, kcal/kg	4610	4750
Total P, %	0.78	0.44
Total Ca, %	2.27	1.31

[1]Supplied per kilogram of diet: manganese (manganese sulphate and manganous oxide), 100 mg; zinc (zinc oxide), 80 mg; iron (ferrous sulphate), 20 mg; copper (copper sulphate), 10 mg; iodine (calcium iodate), 1 mg; molybdenum (sodium molybdate), 0.48 mg; selenium (sodium selenite), 0.25 mg; folic (folic acid), 1.5 mg; vitamin A (retinyl acetate), 13.5 mg; vitamin E (d1-α tocopherol acetate), 100mg; vitamin D_3 (cholecalciferol), 5mg; vitamin B_1 (thiamine mononitrate), 3mg; vitamin B_2 (riboflavin), 10mg; vitamin B_3 (niacinamide), 60 mg; vitamin B_5 (calcium panthothenate), 15 mg; vitamin B_6 (pyridoxine HCl), 3mg; vitamin B_{12} (cyanocobalamin), 30mg; vitamin K (menadione sodium bisulphate complex), 5.0 mg; biotin (biotin), 125 mg.

Table 2. Acid-binding capacity (ABC) and buffering capacity (BUF) of the experimental diets

	High limestone diet[2]	Low limestone diet[3]
pH[3]	6.31	6.29
SEM	0.04	0.09
ABC-2[4]	5200	4000
ABC-3[5]	2900	2600
ABC-4[6]	1600	1550
SEM	70.7	75.0
BUF-2[7]	1203	860
BUF-3[8]	887	747
BUF-4[9]	675	597
SEM	26.5	40.0

[3]Initial pH of samples

[4]Acid-binding capacity to pH 2

[5] Acid-binding capacity to pH 3

[6] Acid-binding capacity to pH 4

[7] Buffering capacity to pH 2

[8] Buffering capacity to pH 3

[9] Buffering capacity to pH 4

578 **Table 3.** Influence of dietary calcium level and bird age on
579 in situ gizzard pH of broilers (Experiment 1)[1]

Age, day	High limestone diet[2]	Low limestone diet[3]	No. birds required to predict dietary differences[4]
7	2.42[c]	2.33[d]	10
14	2.71[b]	2.42[c]	17
21	1.88[e]	1.86[e]	28
28	2.18[e]	2.26[d]	22
35	3.84[a]	2.22[e]	9
42	2.47[c]	3.59[a]	6
SEM		0.20	
Diet x age		<0.001	

580 [1] Means represent the average of 8 birds per day, 48 birds total, with 4 birds per diet each
581 day.
582 [2] High limestone diet contains 0.80% monocalcium phosphate and 2% limestone.
583 [3] Low limestone diet contains 0.40% monocalcium phosphate and 1% limestone.
584 [4]Number of birds necessary to predict differences between the High limestone and Low
585 limestone effect on gizzard pH, based on statistical power calculation
586 [a-e] Means with no common superscript are different ($P < 0.05$)

587 **Table 4.** Influence of dietary calcium level, bird age, method[4] and digesta temperature on gizzard and digesta pH of broilers measured ex situ over
588 a 3 minute time period (Experiment 2)[1]

Age, day	High limestone diet[2]				Low limestone diet[3]		
		Ex situ				Ex situ	
	In situ	Ambient temperature, 14°C	Water bath, 41°C		In situ	Ambient temperature, 14°C	Water bath, 41°C
Gizzard							
7	2.42^{f}	2.65^{e}	2.40^{f}		2.33^{f}	2.56^{e}	2.37^{f}
14	2.71^{d}	2.84^{d}	2.78^{d}		2.42^{e}	2.69^{e}	2.60^{e}
28	2.18^{g}	2.27^{f}	2.19^{g}		2.26^{g}	2.31^{f}	2.21^{g}
SEM				0.079			
Diet x age				0.017			
Temperature x age				0.001			
Temperature x method x diet				0.003			
Duodenum							
7	5.89^{c}	5.98^{c}	5.96^{c}		5.80^{c}	6.12^{bc}	5.98^{c}
14	6.14^{b}	6.24^{a}	6.16^{b}		6.10^{b}	6.26^{a}	6.15^{b}
28	5.93^{c}	5.81^{c}	5.89^{c}		5.78^{c}	5.82^{c}	5.67^{c}
SEM				0.100			
Temperature x age				0.048			
Method				0.033			

589 [1] Means represent the average of 8 birds per day, 24 birds total, with 4 birds per diet each day.
590 [2] High limestone diet contains 0.80% monocalcium phosphate and 2% limestone.
591 [3] Low limestone diet contains 0.40% monocalcium phosphate and 1% limestone.
592 [4] pH measured in situ or at 75 s (the highest exponential point) after the digesta had been removed from the tract.
593 [a-e] Means with no common superscript are different ($P < 0.05$).

Table 5. Correlations and relative contributions of the effect of time exposed to air, temperature, and dietary limestone on digesta pH and dietary limestone effect on gizzard and duodenal digesta pH of broilers (Experiment 2)[1]

	Gizzard pH			Duodenum pH		
Age, day	d 7	d 14	d 28	d 7	d 14	d 28
Relative contributions						
R-square	0.63	0.95	0.93	0.80	0.24	0.49
Beta[2]						
Time[3]	0.02	0.07	0.05	0.11	0.33	0.10
Temperature[4]	0.81	1.56	0.63	2.39	0.65	0.57
Diet[5]	1.40	3.14	1.79	1.26	0.18	0.52
Correlations[6]						
Time	0.02	0.07	0.00	0.02	0.33	-0.05
Temperature	0.39	0.08	-0.21	0.65	0.33	-0.26
Diet	-0.37	-0.40	0.13	0.39	0.10	-0.52

[1] Represent the average response of 8 birds per age, 24 birds in total, 4 birds on each diet at each sampling point.
[2] Coefficient to indicate statistically significant unique contribution of the factor.
[3] Log time of seconds digesta was exposed to air post removal from the tract (15 to 180 s).
[4] Digesta measured at either room temperature (14.4°C) or at 41°C.
[5] Digesta of birds fed either the high limestone diet (0.8% monocalcium phosphate and 2% limestone) or low limestone diet (0.4% monocalcium phosphate and 1% limestone).
[6] Correlations between factor and pH readings.

606 **Table 6.** Influence of method[2] and bird age on gizzard and duodenum pH of broilers
607 (Experiment 3)[1]

	Gizzard		Duodenum	
Age, days	In situ	Reconstituted[3]	In situ	Reconstituted[3]
21	2.14	2.65	5.87	6.39
35	2.92	3.51	6.17	6.48
42	3.04	3.73	6.05	6.29
SEM	0.18		0.06	
Method	<0.001		<0.001	
Bird age	<0.001		0.059	

608 [1] The mean represents the average of 12 birds per age, 36 birds in total, 6 birds on
609 each diet at each sampling point.
610 [2] pH measured in situ or in samples that had been standardised with a known
611 volume of water.
612 [3]Digesta samples that had been removed from the tract, snap frozen, freeze dried
613 and reconstituted with volume of water equal to the average of all samples collected
614 for that section of tract.

615 **Table 7.** Correlations and relative contribution of reconstitution[3] and dietary limestone on
616 gizzard and duodenal digesta pH of broilers (Experiment 3)[1]

	Gizzard pH			Duodenum pH		
Age, day	d 21	d 35	d 42	d 21	d 35	d 42
Relative contributions						
R-square	0.63	0.36	0.10	0.53	0.40	0.17
Beta[2]						
Reconstitution[3]	0.50	0.45	0.33	0.60	0.54	0.41
Diet[4]	0.82	0.70	0.04	0.47	0.48	0.35
Correlations[5]						
Reconstitution	0.51	0.49	0.33	0.66	0.57	0.36
Diet	-0.20	-0.35	0.04	-0.17	-0.30	0.22

617 [1] Represent the average response of 12 birds per age, 36 birds in total, 6 birds on each diet at
618 each sampling point.
619 [2] Coefficient to indicate statistically significant unique contribution of the factor.
620 [3] Digesta samples that had been removed from the tract, snap frozen, freeze dried and
621 reconstituted with volume of water equal to the average of all samples collected for that
622 section of tract.
623 [4] Digesta of birds fed either the high limestone diet (0.8% monocalcium phosphate and 2%
624 limestone) or low limestone diet (0.4% monocalcium phosphate and 1% limestone).
625 [5] Correlations between factor and pH readings.