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# Effects of the dietary supplementation of organic acids on gut health in growing rabbits

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**RIASSUNTO**

Gli acidi organici sono stati usati con successo per molti anni sia nei polli da carne che nei suinetti. In entrambe le specie, combinazioni di acidi organici sono state utilizzate per ridurre il pH dello stomaco, per ottenere effetti favorevoli sul microbiota. Alcuni di questi acidi organici sviluppati per altre specie sono stati spesso utilizzati negli allevamenti di conigli da carne. Tuttavia, la loro efficacia non è ancora chiara. Tra i pochi studi esistenti, alcuni hanno osservato effetti positivi con l'introduzione di alcune combinazioni di acidi commerciali, ma altri studi hanno osservato un aumento della mortalità durante il periodo di crescita. La fisiologia digestiva del coniglio presenta particolarità, come la presenza del ciecotrofo o il basso livello di lattosio nel latte, che rendono il comportamento del pH gastrico diverso dalle altre specie.

L'obiettivo dell'esperimento è stato valutare in conigli svezzati a 28 giorni di età il comportamento di specifici acidi organici a diverso pH, per valutare i loro effetti sulle prestazioni e sul pH gastrointestinale. Lo scopo principale dell'acidificazione del tratto gastrointestinale è quello di creare una barriera contro gli agenti patogeni e ottimizzare la digestione delle proteine.

Per raggiungere questi scopi, 240 conigli sani ambo sessi sono stati divisi in 2 lotti di 120 conigli ciascuno. Lo studio si è protratto per 7 giorni, a partire dal ventottesimo giorno di età dei conigli e si è concluso al trentacinquesimo giorno di età. 6 acidi organici (acido acetico, formico, propionico, lattico, citrico e butirrico) a dosi diverse per raggiungere il pH 3, pH 4 e pH 5 sono stati disciolti in acqua e confrontati con acqua di controllo (acqua convenzionale non acidificata a circa pH 7). Per ogni lotto i conigli sono stati suddivisi in 20 gabbie (6 animali/gabbia): 18 gabbie fornite di acidi organici + 1 gabbia fornita con l’acqua di controllo + 1 gabbia per il sacrificio iniziale. Tutti gli animali provenienti dalla gabbia del sacrificio iniziale sono stati macellati a 28 giorni di età. In seguito, metà degli animali sono stati macellati a 31 giorni di età (3 per trattamento) e l'altra metà a 35 giorni di età (3 per trattamento) per registrare il pH dello stomaco (sia nel fundus che nell'antrum) e dell'intestino tenue (a livello del duodeno, digiuno, ileo e cieco).

I risultati ottenuti hanno mostrato che la somministrazione di acidi organici attraverso l'acqua non ha migliorato le prestazioni di crescita dei conigli in modo significativo, ma ha avuto un effetto sui valori del loro pH gastrointestinale. I trattamenti con pH 3 tendevano ad abbassare il peso corporeo, l’aumento di peso medio giornaliero e l'assunzione media giornaliera di mangime e acqua, mentre i trattamenti a pH 4 e 5 non hanno indotto differenze rispetto all’acqua di controllo, né in modo positivo né negativo. La somministrazione degli acidi organici si è dimostrata più efficace nel tratto intestinale piuttosto che nello stomaco, dove l’effetto tampone del mangime ha influenzato maggiormente l'efficacia degli acidi. Estendere la durata di integrazione degli acidi potrebbe portare a risultati diversi ed essere la chiave per migliorare le prestazioni di peso e lo stato di salute dei conigli (agendo sul loro pH intestinale). Tuttavia, è necessario considerare il limitato sviluppo fisiologico del tratto gastrointestinale dei conigli allo svezzamento e il modo in cui questa condizione potrebbe influire sull'efficienza digestiva.

Somministrare gli acidi organici ai conigli d'allevamento tramite l'acqua bevuta è un modo strategico per aiutare uno sviluppo gastrointestinale sano allo svezzamento; e potrebbe avere un certo potenziale nel migliorare le prestazioni se l'integrazione di acidi viene prolungata nel tempo. In ogni caso, sono necessari ulteriori studi che analizzino il comportamento d'assunzione degli acidi e la loro efficacia al momento dello svezzamento, in modo da definire con maggiore precisione il rapporto tra l'assunzione di acidi e lo sviluppo del tratto gastrointestinale nei conigli d'allevamento.

## ABSTRACT

Acidifiers have been used successfully for many years in both broilers and piglets. In both species, combinations of organic acids are used to reduce the pH of the stomach, which sometimes has favourable effects on the microbiota. Some of these acid mixtures developed for other species are being frequently used in rabbit farms. However, their effectiveness is still not clear. Among the few existing studies, some have observed positive effects with the introduction of some commercial acid mixtures, but others have observed an increase in mortality during the growing period with their use. The digestive physiology of the rabbit has peculiarities that make the behaviour of its gastric pH different from other species, as the presence of soft faeces or the low level of lactose in milk.

The objective of the experiment is to evaluate the behaviour of specific organic acids in weaned rabbits at different pH to assess their effects on performance and gut pH. The main purpose of acidification of the gastrointestinal tract is to create a barrier against pathogens and optimizing protein digestion.

To reach these purposes, a total of 240 healthy rabbits from both sexes have been divided into 2 batches of 120 rabbits each. The trial started at 28 days of age and ended at 35 days of age (7 days).

A total of 6 organic acids (acetic, formic, propionic, lactic, citric and butyric) at different doses to achieve pH 3, pH 4 and pH 5 were dissolved in water and compared with a control (non-acidified conventional water at around pH 7). For each batch rabbits have been divided in 20 cages (6 animals/cage): 18 cages provided with organic acids treatments + 1 cage for control water + 1 cage for the initial sacrifice. All animals from the Initial sacrifice cage were slaughtered at 28 days of age. Then, half of the animals were slaughtered at 31 days of age (3 per treatment) and the other half at 35 days of age (3 per treatment) to register the pH of stomach (both in fundus and antrum) and small intestine (at duodenum, jejunum, ileum and caecum).

Obtained results showed that the administration of organic acids via drinking water did not improve growth performance of weaned rabbits in a significative way, but had effect on their gastrointestinal pH values. Treatments with pH 3 tended to lower down body weight, average daily gain and average daily feed and water intake, while treatments at pH 4 and 5 did not induce differences compared to the control water, either in a positive or negative way. Acids administration was more effective in the intestinal tract rather than in the stomach, where buffering capacity of feed most influenced the effectiveness of acids.

Extending the supplementation duration of acids during fattening could lead to different results and be the key to enhance weight performance and health status of rabbits. However, the limited physiological development of the gastrointestinal tract of rabbits at weaning and how this condition could impact digestive efficiency have to be considered.

Supplementing acids in rabbit farming by water is a strategical way to help a healthy gastrointestinal development at weaning phase; and could have some potential in enhancing performance if acids supplementation is prolonged through time. Further studies on behaviour of acids' intake and

effectiveness at weaning period in rabbit farming are necessary to define more precisely the relationship between acid intake and GIT health status and development.

# 1. INTRODUCTION

## *1.1 Panoramic on rabbit's digestive system*

The European rabbit (Oryctolagus cuniculus L.) is a native of the Iberian Peninsula (the first fossils were found in Huelva, Spain) (Zeuner, 1963). The rabbit is an herbivore, or more specifically a folivore, designed to exist on a diet of green vegetation. Due to its origins, the rabbit developed a digestive system capable of using the flora available in the Mediterranean Basin, characterized by its richness in graminaceous plants, bushes, shrubs and grasses rich in protein (Ferreira and Célio Alves, 2009). However, the small size of the rabbit means it has a correspondingly high metabolic rate. To cope with this problem the rabbit has evolved a digestive tract radically different to that of the better-known herbivores such as the horse (a colon fermenter) and the ruminants (gastric fermenters). The rabbit has a system that: (1) allows a high food intake, (2) separates out the digestible and easily fermentable components of the diet, and (3) rapidly eliminates the slowly fermentable fibrous waste that would otherwise have to be carried around. The separation of fermented and non-fermented components of the diet is centred around the caecum, which is in the need of a consistent diet high in long particle length (>0.5 mm) and indigestible fibre to maintain its motility. The rabbit's digestive system also eliminates the need for having a large absorptive surface area in the large intestine by complete separation of the products of caecal fermentation (caecotrophs) and the faeces, allowing reingestion and absorption of bacteria and their by-products in the small intestine. Given that the system is geared for the rapid elimination of fibrous wastes, it is somewhat curious that the main driving force for the system is the presence of such indigestible fibre. Lack of this fibre is one of the most common causes of gastrointestinal disturbance in rabbits (Rees Davies *et al.*, 2003).

## *1.2 The behaviour of Caecotrophy*

Caecotrophy is a complete behaviour involving the excretion and immediate consumption of specific faeces, named soft faeces or 'caecotrophes'. Consequently, the daily intake behaviour of the rabbit is comprised of two meals: feeds and caecotrophes. Caecotrophy plays an important role in rabbit nutrition, providing proteins and B vitamins from bacterial sources. Caecotrophy behaviour starts around 21–25 days of age, when a significant solid feed intake occurs, leading to both caecal and colon filling (Gidenne *et al.*, 2002a; Orengo and Gidenne, 2007) and to an increased microbiota activity. Hard faecal pellets are excreted, but soft faecal pellets are totally recovered by the rabbit directly upon being expelled from the anus. To do this the rabbit twists itself around, sucks in the soft faeces as they emerge from the anus and then swallows without chewing them.

## *1.3 Gastrointestinal anatomy and physiology of the rabbit*

A schematic diagram of the anatomy of the digestive tract of the rabbit is provided for reference in *Figure 1.1*, and an overview of the activity of the digestive system is provided in *Figure 1.2*.

### *1.3.1 From ingestion to stomach*

Rabbits utilize the chisel-like incisors to cut off the short pieces of vegetation (Hirschfeld *et al.*, 1973). This is enabled by the long diastema, rostrally positioned incisor teeth and cleft upper lip (''hare-lip''). Once ingested, feed material is ground down by the cheek teeth (premolars and molars).

The esophagus serves as a transport duct from the oral cavity to the stomach and has little or no effect on digestion (Ruckebusch *et al.*, 1991).

The stomach of the rabbit is a thin-walled, pouch-like organ. It comprises 15% of the gastrointestinal tract volume when empty (Brewer and Cruise, 1994). The cardiac portion of the stomach is thin walled, nonglandular, and intrinsically immobile (Brewer and Cruise, 1994). The fundus is the major secretory portion of the stomach, and has parietal cells (which secrete hydrochloric acid and intrinsic factor) and peptic cells (which secrete pepsinogen, the precursor of pepsin). The pyloric region has a much thicker muscular wall. The adult rabbit's gastric pH during digestion of food material is maintained between 1 and 2, which destroys most microbial organisms, maintaining an almost sterile stomach and small intestine (Cheeke, 1987; Fekete, 1989). Passage of feed material through the stomach has been estimated to take 3-6 hours (Carabaño *et al.*, 1998). Hydrolysis of proteins begins in the stomach, with the pepsin-HCl complex. An important exception is the digestion of the mucin covering the caecotrophs. The caecotrophs are not macerated by the teeth, and remain intact, protected by their mucinous coat, within the stomach for at least 6-8 hours after ingestion (Carabaño *et al.*, 1998). During this time, the caecal material within the caecotrophs is protected from the adverse gastric pH, and microbial fermentation continues, leading to lactic acid formation within the stomach (Griffiths and Davies, 1963). The pH in the stomach during the period when caecotrophs are present increases to 3 due to the buffering effects of lactate produced by microbes in the caecotrophs (Lang, 1981).

### *1.3.2 Small intestine*

Gut motility can be divided into two different processes. Segmentation is the process involved in the mixing of intestinal contents by periodic static constriction of the intestinal wall and is particularly important in the rabbit duodenum. Peristalsis is a different process and involves a ring of contraction moving gradually along the intestine, usually in an aboral direction. Peristaltic contractions occur slowly every 10–15 minutes, and do not alter with the stages of the cecotrophic cycle. Small intestinal motility in the rabbit, as in the human, is regulated in part by motilin, a peptide secreted by enterochromaffin cells of the duodenum and jejunum. Motilin stimulates smooth muscle contractions (Ito, 1990). At the distal end of the ileum, dorsal to the large intestine in the left caudal abdominal quadrant, there is a round, muscular ampulla referred to as the sacculus rotundus (Donelly, 1997; Jenkins, 2000). This structure has an immunological function and is only found in lagomorphs (Jenkins, 2000). The ileocecal valve, sited between the ileum and the sacculus rotundus, reverses flow of fluid into the ileum and directs chyme via the sacculus rotundus to the cecum (Jenkins, 2000).

The pancreas of the rabbit is small, diffuse and often difficult to locate within the mesenteric fat located between the colon, stomach, and duodenum. Trypsin, chymotrypsin, and carboxypeptidases are produced in the pancreas and released into the intestinal lumen to complete protein digestion. Lipases of various forms are also produced. The pancreas is an important source of bicarbonate ions that neutralize the acidic chyme entering the small intestine from the stomach.

The liver synthesizes and releases bile acids into the small intestine where a proportion of these is converted by microbial activity to deoxycholic acid (Cheeke, 1987). The bile acids are important as detergents that break down fatty or oily material into small micelles, allowing absorption of fats and fat-soluble vitamins in the distal small intestine.

Small intestinal digestion and absorption in the rabbit are similar to that in other species: bicarbonate ions are secreted in the duodenum to neutralize the acidity of the chyme passing from the stomach. Most of the digestion of carbohydrates and simple proteins takes place in the duodenum and jejunum and the products of this digestion (monosaccharides, amino acids) are absorbed across the jejunal brush border. The ileum also plays an important role in regulating and recycling the electrolytes secreted by the stomach and proximal small intestine by reabsorbing bicarbonate ions.

### *1.3.3 Large intestine*

The rabbit's cecum represents 40–60% of the total volume of the gastrointestinal tract (Jenkins, 2000). It is a blind sac that folds into four parts (gyri). The first three gyri have thin, translucent walls. The ''vermiform'' appendix forms the final fold. It is a 45 cm blind tube that ends on the left flank dorsal to the first part of the cecum and has thick walls containing lymphoid tissue (Donelly, 1997; Barone *et al.*, 1973; Brooks, 1997). The appendix secretes bicarbonate ions into the cecal lumen, which act as a buffer for the volatile fatty acids produced by cecal fermentation (Cheeke, 1987; Williams *et al.*, 1961). Water, secreted by the appendix and colon, is continually added to the cecal contents from where it is absorbed across the cecal wall. This maintains a soft paste to viscous liquid consistency of the cecal contents (Harcourt-Brown, 2002). The normal pH of the rabbit cecum varies with the stage of the cecotrophic cycle, however, during the most acidic period of the day is generally of 5.9-6.8 points in adult rabbits and 5.4-6.3 point in weaning rabbits (Lelkes and Chang, 1987). The cecum provides an anaerobic fermentation chamber for organisms such as *Bacteroides spp.*, which are found at up to $10^9$/g (Fekete, 1989).

The colon of the rabbit is divisible into several different positional and morphologic parts. The ascending colon is very long and divided into five limbs extending forwards and back separated by flexures. The first limb has three taeniae forming three rows of sacculations or haustrae. Second and third limbs there are a single taenia and one row of haustra. The remainder of the ascending colon has no taeniae and lies coiled in the dorsal part of the abdominal cavity. The transverse colon is short, and ends in a muscular thickening known as the fusus coli, a structure unique to lagomorphs. The fusus coli regulates the separation (by contractions of the taeniae/haustrae) of digestible material from indigestible

material and fiber (Ruckesbusch and Fioramonti, 1976). After the fusus coli, the descending colon and rectum return to simple tubular form with thicker walls.

### *1.4 Cecotroph production*

Rabbit's digestive process is the regulation of colonic and cecal motility to allow the separation of intestinal contents into indigestible wastes and fermentable substrates. The process is regulated by motility in the colon and can be broadly subdivided into two ''phases': the ''hard feces phase,'' and the ''soft feces phase'' or ''cecotroph production.''

During the ''hard feces'' phase small intestinal material derived from ingested feed passes through the ileocecal valve and sacculus rotundus. Little cecal fermentation occurs at this stage and the cecum contracts, expelling most of its contents into the proximal colon. Water is secreted by the proximal colonic wall, which aids the processes of mixing and separation of the contents. Under the control of the fusus coli, three separate contraction types occur (Harcourt-Brown, 2002). There is a progressive monophasic peristaltic wave of 5 seconds duration, and a segmental low-frequency 14-second duration contraction (Harcourt-Brown, 2002; Ehrlein *et al.*, 1983). These both progress in an aboral direction. The third contraction type is that of the haustrae, which undergo high-frequency contractions of 3-second durations, which repeatedly churn the colonic contents (Harcourt-Brown, 2002). During this churning process the indigestible fibrous particles of greater than 0.5 mm length accumulate within the central lumen of the proximal colon (Bjornhag, 1972; 1981), while smaller particles are moved to the periphery where they congregate in the pocket-like haustrae (Bjornhag, 1972; 1981). The central fibrous material passes rapidly distally and is formed into ''hard feces'' by the physical compressive actions of the fusus coli. Further, water, electrolyte, and volatile fatty acid absorption occurs as the pellets pass through the distal colon, and they are finally expelled as small, dry, hard fecal pellets. They do not have a mucus covering. Fermentable components and fluid, which have accumulated in the haustrae, are passed by retrograde peristalsis back up the colon and into the cecum for fermentation (Bjornhag, 1981). Following fermentation, the cecal contents form a soft dark green paste, which is rich in semi-digested food material as well as microbial organisms. The contractions that functioned to maintain the separation of fluid and different-sized particles during the hard feces phase now decrease. Peristaltic monophasic contractions increase, occurring every 1.5 seconds (Harcourt-Brown, 2002). The cecum contracts and the cecal contents are passed rapidly through the colon. Colonic transit time during the ''soft feces'' phase is reportedly 1.5-2.5 times faster than during the ''hard feces'' phase (Ruckesbusch and Fioramonti, 1976). The fusus coli contractions during cecotroph production are gentler, and do not expel the fluid from the pellets. Goblet cells in the fusus secrete mucus. As the cecotroph pellets pass through the distal colon lysozyme is added, and the pellets are coated with mucus. Cecotrophs arrive at the anus and are ingested directly in bunches as a response to a number of factors, including rectal mechanoreceptor stimulation, olfactory stimuli, and blood concentrations of various metabolites and hormones (Harcourt-Brown, 2002; Fekete, 1989).

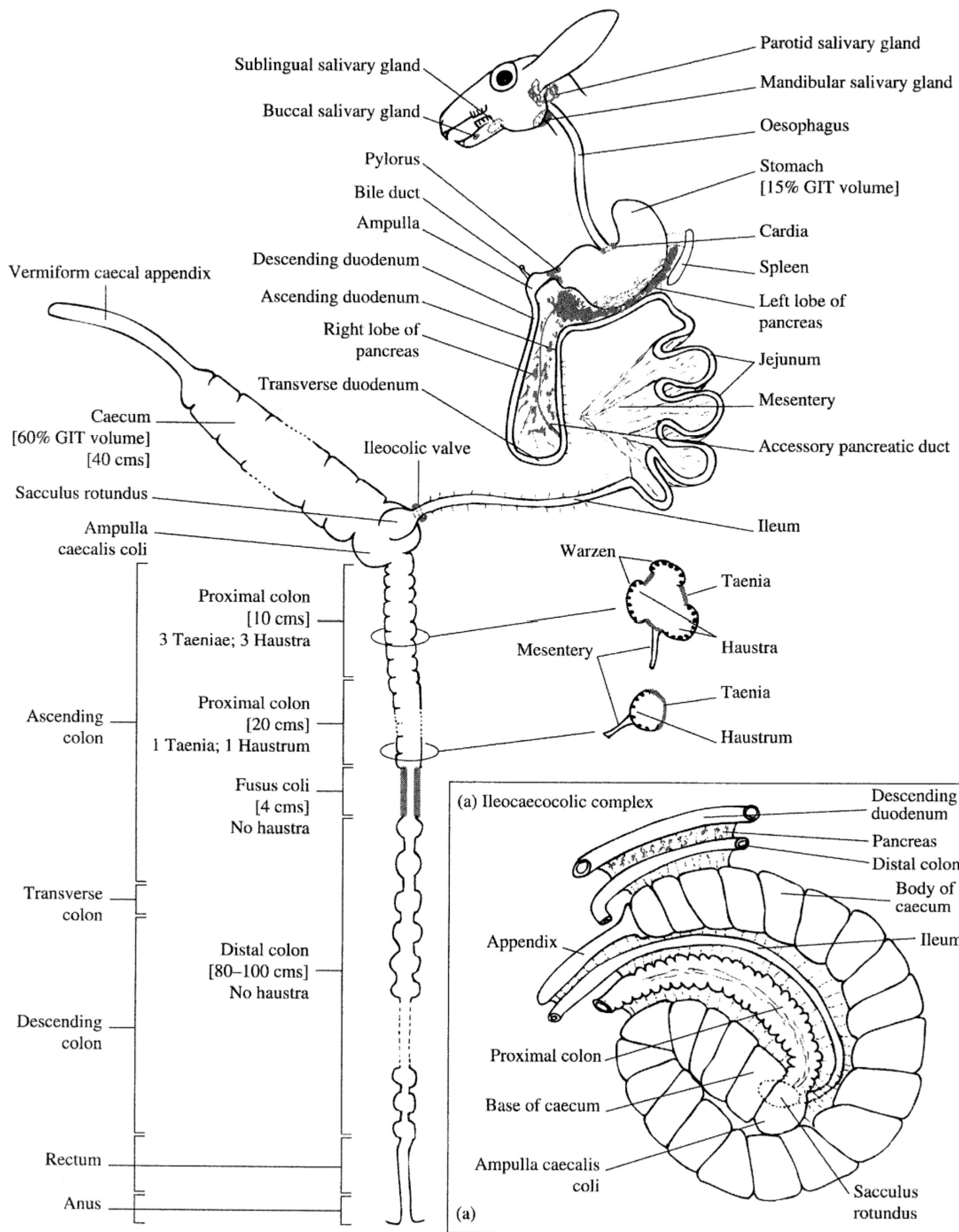


*Figure 1.1* - Schematic diagram of the anatomy of the alimentary tract of the rabbit. (From Harcourt-Brown F. Textbook of rabbit medicine. Oxford: Butterworth-Heinemann; 2002.)

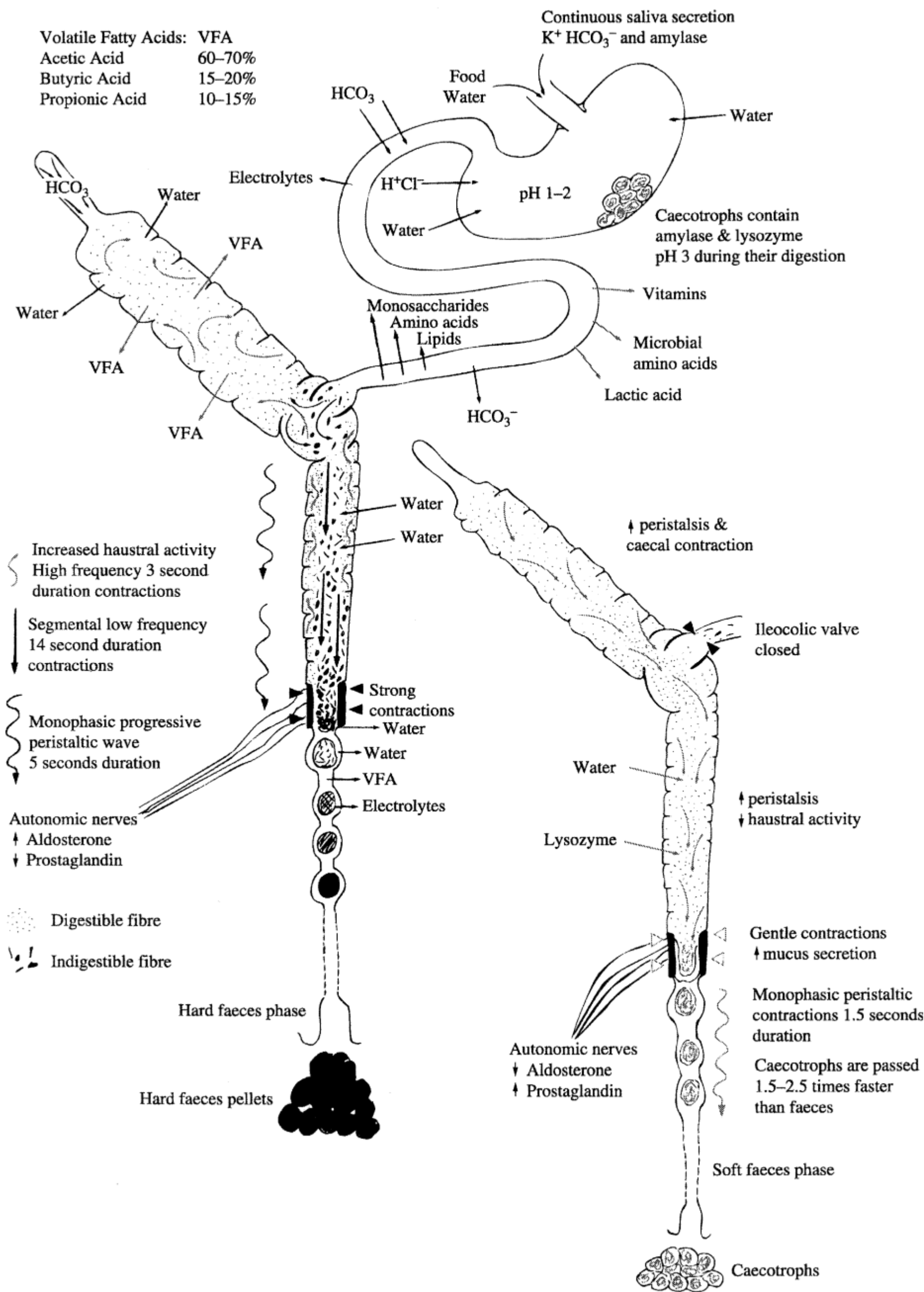


*Figure 1.2* - An overview of the activity of the digestive system of the rabbit. (Adapted from Harcourt-Brown F. Textbook of rabbit medicine. Oxford: Butterworth-Heinemann; 2002.)

## 1.5 Feeding behaviour of the young rabbit - from milk to solid food

Females give birth to naked and blind young in a nest after 31 days of gestation. There is subsequently a period of rapid development for the youngs, ending in weaning around 1 month later. During this period, kits progress from a single milky meal a day (until 14 days of age), to several solid meals a day (Gidenne *et al.*, 2020).

*1.5.1 Milk intake*

In general, rabbit milk can be characterised as high in protein (12.3%) and fat (12.9%) with low lactose content (1.7%). Compared to cow and sow milk, rabbit milk is respectively 2 and 3 times more concentrated in fat and protein. The lactose content is only one-third of the two other species. Remarkable is also the high energy content of rabbit milk (8.4 MJ/kg) which explains the rapid growth of the kits (Maertens *et al.*, 2006).

During the first week post-partum, kits drink about 0.15 of their live weight (LW) in milk each day in one nursing session, and up to 0.25 for some individuals (Lebas, 1969). Between 4 and 6 days of age kits also consume some hard faeces left by the doe in the nest, thus stimulating the caecal microbiota maturation (Kovács *et al.*, 2004). Thereafter, individual milk intake increases gradually to reach a peak of about 25 g day$^{-1}$ between 17 and 25 days of age (*Figure 1.3*). During this period, milk intake is highly variable between kits due to individual ability, competition between littermates and milk availability (Fortun-Lamothe and Gidenne, 2000). After day 20–25, maternal milk production progressively decreases. In commercial systems, weaning is generally carried out between 28 and 35 days of age, even if milk production has not completely stopped.

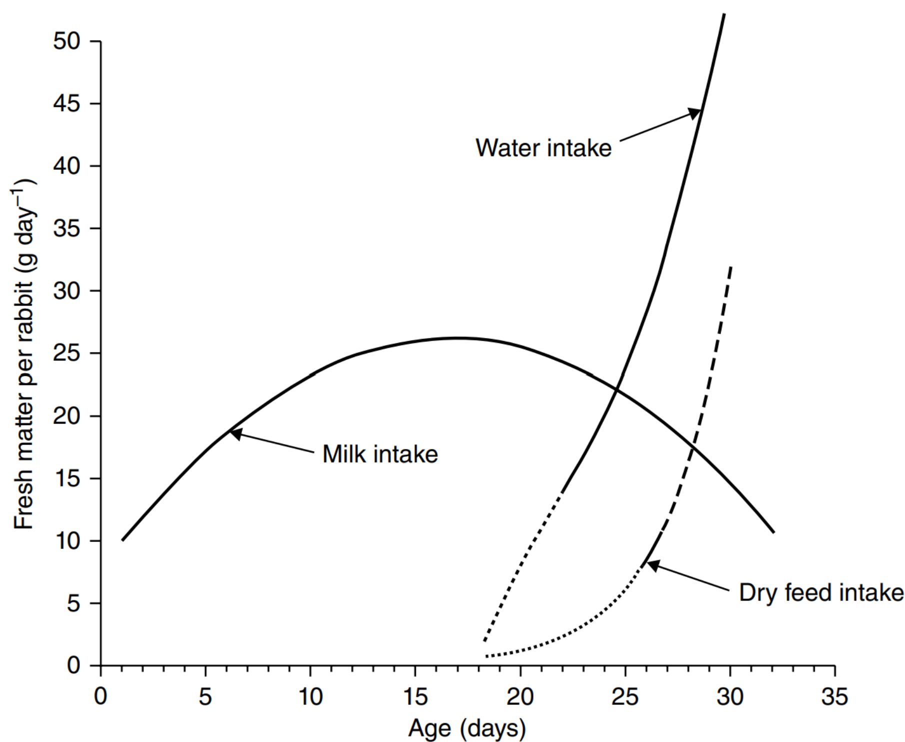


*Figure 1.3* - Milk, water and dry feed intake of the young rabbit before weaning in a conventional farming system. Values are means for litters of seven to nine kits reared in a conventional system (pelleted feed, nipple drinking, weaning at 30 days, does mated 11 days after kindling).
(Adapted from Szendrö *et al.*, 1999; Fortun-Lamothe and Gidenne, 2000).

*1.5.2 Solid feed intake and evolution of nutrient and energy supply*

Young rabbits begin to eat significant quantities of solid feed offered outside the nest at around 16–18 days of age, when they are able to leave the nest and move easily to access a feeder (with pelleted feed) and drinker. Initially, young rabbits eat very small quantities of pelleted feed (<2 g day$^{-1}$ per rabbit before 20 days of age), increasing from 25 days of age to reach 40–50 g day$^{-1}$ by 35 days (Gidenne *et al.*, 2002b), although this is highly variable between litters.

In parallel to modifications in feeding behaviour, the nutrients intake changes sharply between birth and weaning (*Figure 1.4*). While rabbit milk is very rich in lipids and proteins (Maertens *et al.*, 2006), on the other hand, pelleted feed mainly contains carbohydrates (80 g 100 g$^{-1}$, with varying digestibility ranging from very high for starch to low for fibre), some protein (15–18 g 100 g$^{-1}$) and only a small quantity of lipids (2–5 g 100 g$^{-1}$), all of vegetable origin. The ingestion of vegetable proteins becomes equal to that from the milk at around 25 days of age, and then exceeds it within a few days, while lipids come mainly from milk until weaning. Carbohydrates intake is almost null (<0.3 g day$^{-1}$) until 17 days of age, and becomes significant from day 21 in the form of fibre and starch. However, proteins and fats in the milk constitute the main sources of energy until weaning (Gidenne *et al.*, 2020). Therefore, digestive capacities must evolve rapid, in parallel with the evolution of feeding patterns (Gidenne and Fortun-Lamothe, 2002). According to Wu (1986) secretion of hydrochloric acid begins at 16 days of age and is completely established by 30 days, after which the gastric pH does not change. The gastric pH falls sharply during the period around weaning (*Figure 1.5*); the stomach pH, which is high at 23 days (>5) is still 4-6 at weaning (29 days) and then falls to 1-8 at 36 days and to 1-6 at 42 days. Apart from the possible effect of weaning age or feeding, these variations might be due to the location of measurement: when the rabbit practises caecotrophy, the pH of the fundus is almost neutral while the caecotrophes are being stored, whereas the pH of the antrum always remains very acid (<3 after weaning) (Gidenne and Fortun-Lamothe, 2002).

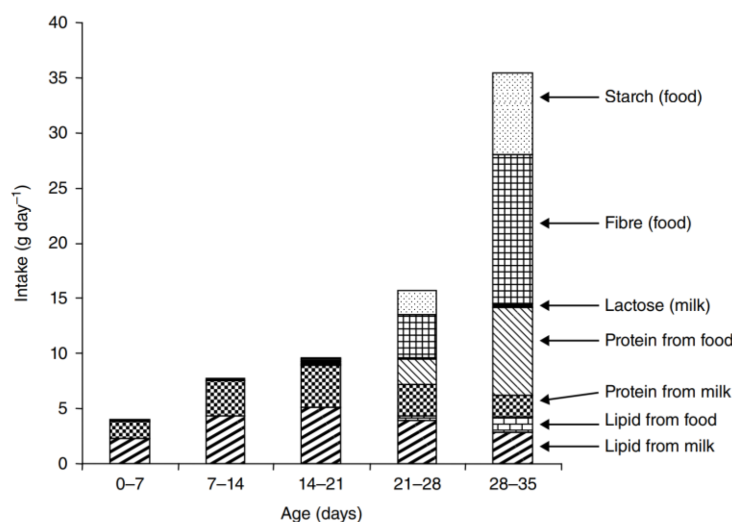


*Figure 1.4* - Changes in nutrient intake of young rabbits between birth (day 0) and weaning (day 35) in conventional farming system (Gidenne *et al.*, 2020).

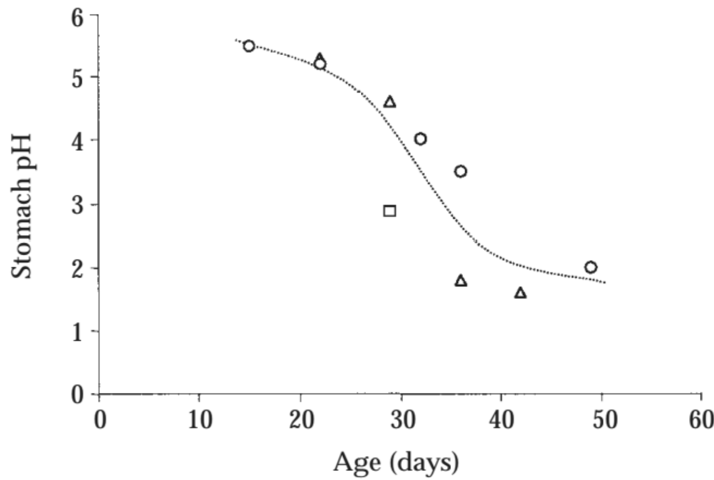


*Figure 1.5* - Stomach pH according to the age of the young rabbit (from: Gidenne and Fortun-Lamothe, 2002).

## *1.6 Feeding behaviour of the growing and adult rabbit*

From weaning (classically between 4 and 5 weeks), the daily feed intake of the domestic rabbit (fed a complete pellet feed) increases in relation to live weight (*Figure 1.6*) and stabilizes at about 5 months of age. The intake of soft faeces increases only until 2 months of age and then remains steady (*Figure 1.6*). Expressed as dry matter, the intake of soft faeces increases from 10 g day$^{-1}$ (1 month old) to 55 g day$^{-1}$ (2 months), thus representing 0.15– 0.35 of the feed intake (Gidenne and Lebas, 1987).

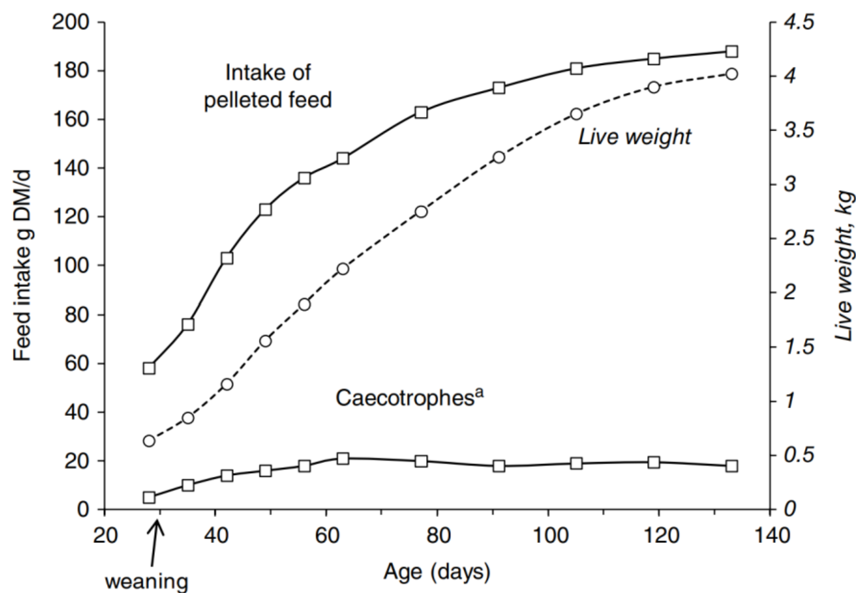


*Figure 1.6* - Intake from feed and caecotrophes, and rabbit growth from weaning (28 days) until adulthood. Data are based on a pelleted feed fed ad libitum regime (Gidenne and Lebas, 1987).

Between weaning (4–5 weeks) and 8 weeks of age, weight gain is at its highest (*Table 1.1*) and feed conversion is lowest. The rate of increase of feed intake and growth rate subsequently decreases, with intake stabilizing at around 12 weeks of age for current hybrid lines of domestic rabbit. Similarly to other mammals, the rabbit regulates its feed intake according to energy requirements (Gidenne *et al.*, 2020).

*Table 1.1* - Feeding behaviour of the domestic rabbit after weaning. Mean values from rabbits (current commercial lines) fed a pelleted diet (890 g dry matter $kg^{-1}$) ad libitum and with free access to drinkable water (Gidenne *et al.*, 2020).

| **Age (weeks)** | 5-7 | 7-10 |
|---|---|---|
| **Solid feed intake (g $day^{-1}$)** | 100-120 | 140-170 |
| **Weight gain (g $day^{-1}$)** | 45-50 | 35-45 |
| **Feed conversion** | 2.2-2.4 | 3.4-3.8 |

The intake pattern of domestic rabbits fed ad libitum comprises several small meals over a 24-h period (Bellier *et al.*, 1995; Gidenne *et al.*, 2010). This meal fractionation is probably linked to the relatively weak storage capacity of the stomach. The amount of ingested feed is not constant throughout the day, increasing during the afternoon, reaching a maximum during the evening and night hours, and becoming minimal in the morning (Gidenne *et al.*, 2010; Birolo *et al.*, 2020a). Water intake increases in parallel to that of feed, and less time is spent drinking than eating. Furthermore, at any age, feeds containing > 0.70 water, such as green forage, provide rabbits with sufficient water at temperatures < 20°C and, in these circumstances, rabbits may not drink at all.

Solid intake fluctuates over a 24-h period (*Figure 1.7*). Over 0.60 of the solid feed (excluding soft faeces) is consumed in the dark period for a domestic rabbit submitted to a 12-h light, 12-h dark schedule (Gidenne *et al.*, 2020). Indeed, feeding behaviour of rabbits shows a low feeding activity during the day (Gidenne *et al.*, 2010; Birolo *et al.*, 2020a), especially in the morning when caecotrophy occurs (Gidenne and Lebas, 2005). The maximum feed intake level (10% of the total daily feed consumption) is observed 2-4 h after the end of the light period and minimum intake 2-4 h after the end of the dark period (Martignon *et al.*, 2009; Birolo *et al.*, 2021). With older rabbits, the nocturnal feeding behaviour becomes more pronounced.

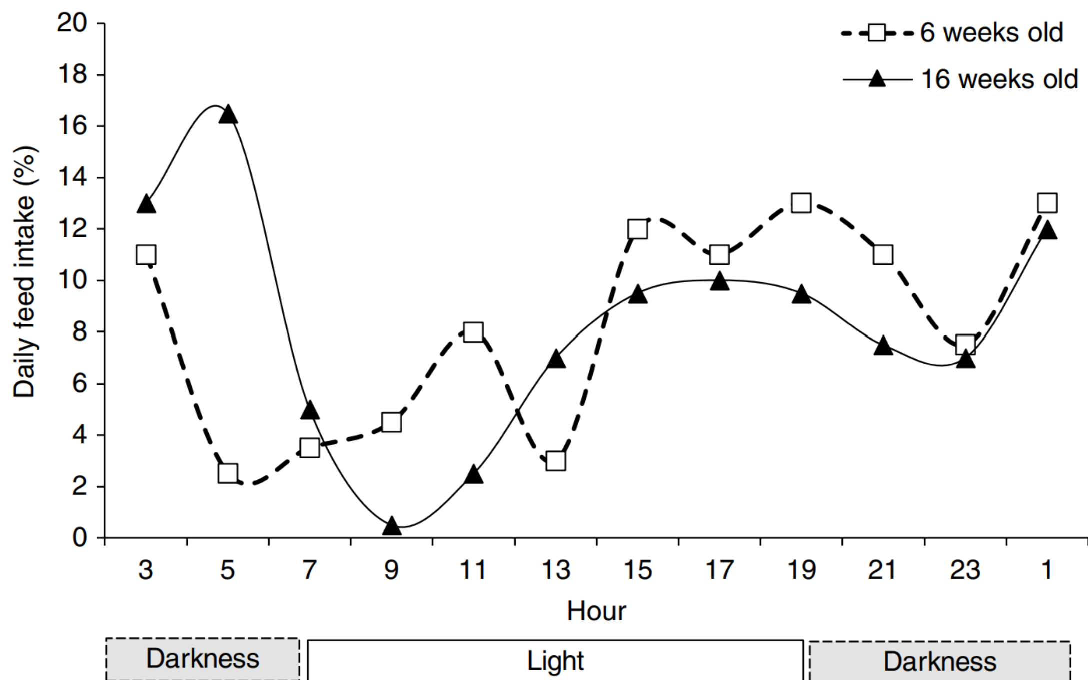


*Figure 1.7* - Circadian pattern of feed intake in the growing or adult rabbit. Mean values for domestic rabbits (n = 6) fed a pelleted feed ad libitum (daily feed intake of 80 and 189 g $day^{-1}$, respectively, for 6- and 16-week-old rabbits) and bred under a 7:00–19:00 light schedule (Bellier *et al.*, 1995).

## *1.7 External Factors Modulating the Feeding Behaviour of the Domestic Rabbit*

### *1.7.1 Ad libitum feeding vs. restricted diet*

Feed restriction can be used as an alternative to ad libitum feeding to reduce mortality and morbidity due to digestive problems in growing rabbits (Birolo *et al.*, 2021; Gidenne *et al.*, 2009, 2012; De Blas *et al.*, 2012) and to enhance farm feed efficiency (Knudsen *et al.*, 2014; Gidenne *et al.*, 2017; Birolo *et al.*, 2020a). Restrictions on diet can be either quantitative or time-based restriction programmes (ranging from 63% to 75% of ad libitum). Both methods showed a positive effect on feed efficiency (Foubert *et al.*, 2007; Knudsen *et al.*, 2014). Along with rabbit feeding behaviour and digestive physiology, feed efficiency in growing rabbits gave better results on nightly restriction programs compared with a daily restriction programme (Birolo *et al.*, 2021; Weissman *et al.*, 2009).

Of course, the effect of feed restriction on the performance and feed efficiency of growing rabbits can vary widely depending on the restriction rate and the duration of both restriction and refeeding phases (Xiccato, 1999; Gidenne *et al.*, 2012).

The caecal volatile fatty acid concentration is higher and caecal pH is lower in restricted rabbits if compared to rabbits fed ad libitum. This condition might limit the proliferation of pathogens in the gut and protect feed-restricted rabbits against digestive diseases (De Blas *et al.* 2012; Gidenne and Feugier, 2009). Time-based feed restriction has been confirmed to affect performance in a negative way at first,

but rabbits quickly adapted their feed intake to the available feeding time. Thus, at the end of the trial, differences among restricted rabbits and fed-ad libitum rabbits disappeared, with no residual effects on slaughter results, carcass, and meat quality (Birolo *et al.*, 2021; Birolo *et al.*, 2020b).

### *1.7.2 Feed composition and presentation form*

One of the main dietary components implicated in feed intake regulation, after weaning, is the digestible energy (DE) concentration. The domestic rabbit (fed a pelleted balanced diet) is able to regulate its DE intake (and thus its growth) when the dietary DE concentration is between 9 and 11.5 MJ $kg^{-1}$, or when the dietary fibre level is between 10% and 25% acid detergent fibre. The intake level is thus well correlated with the dietary fibre level, compared to the dietary DE content (*Figure 1.8*). However, the incorporation of fat in the diet, while maintaining the dietary fibre level, increases the dietary DE level, but leads to a slight reduction in intake. Other nutrients in the diet, such as proteins and amino acids, are able to modify the food intake (Tome, 2004).

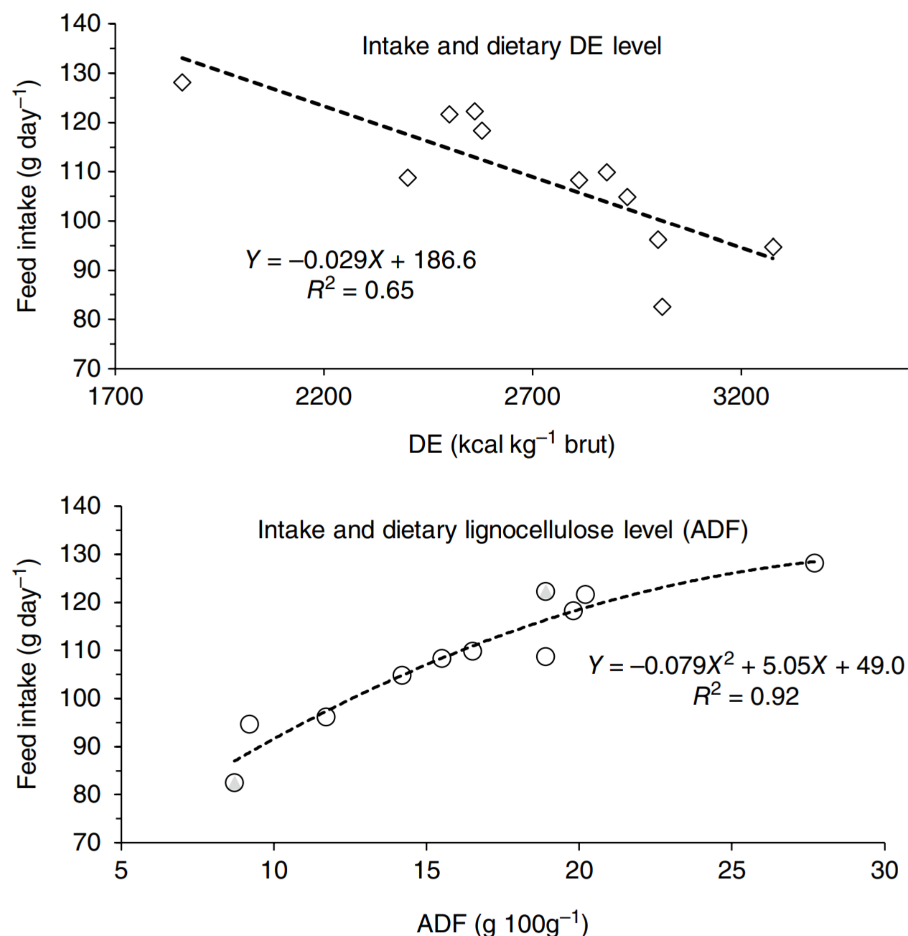


*Figure 1.8* - Feed intake prediction for the growing rabbit, after weaning (between 4 and 11 weeks of age) in conventional farming system (pelleted feed). ADF, acid detergent fibre; DE, digestible energy (Gidenne, 2000).

The presentation of the diet is an important factor that modulates feeding behaviour in the rabbit. Compared to meals, pelleted feeds are preferred at 97% when offered in free choice (Harris *et al.*, 1983). Furthermore, meals seem to modify the circadian cycle of feed intake (Lebas and Laplace, 1977). Pellet size and quality (hardness, durability) also affect feeding behaviour. A reduction in pellet diameter, which also increases the hardness, reduces the feed intake of young and growing rabbits (Maertens, 1994; Gidenne *et al.*, 2003), although the time spent on feeding is increased.

*1.7.3 Environmental factors affecting the feeding behaviour of the rabbit*

Energy expenditure and hence the requirements and feed intake of the rabbit depend on the ambient temperature. Studies on growing rabbits have shown that the intake of pelleted feed drops from 180 to 120 g $day^{-1}$ and water intake rises from 330 to 390 g $day^{-1}$ at temperatures between 5°C and 30°C (*Table 1.2*) (Eberhart, 1980). A closer analysis of feeding behaviour shows that the number of solid meals eaten in 24 h drops as temperature increases, from 37 solid feeds at 10°C to only 27 feeds at 30°C (for 6-week-old New Zealand White rabbits; Prud'hon, 1976). The amount eaten at each meal also decreases with higher temperatures (from 5.7 g per meal at 10–20°C to 4.4 g per meal at 30°C). Water intake increases, however, from 11.4 to 16.2 g per meal between 10°C and 30° C (Prud'hon, 1976).

*Table 1.2* - Feeding behaviour of the growing rabbit according to ambient temperature (Eberhart, 1980).

| **Ambient temperature** | 5°C | 18°C | 30°C |
|---|---|---|---|
| **Relative humidity (%)** | 80 | 70 | 60 |
| **Pelleted feed eaten (g $day^{-1}$)** | 182 | 158 | 123 |
| **Water intake (g $day^{-1}$)** | 328 | 271 | 386 |
| **Water to feed ratio** | 1.80 | 1.71 | 3.14 |
| **Average weight gain (g $day^{-1}$)** | 35.1 | 37.4 | 25.4 |

With no water at all, and depending on temperature and humidity, an adult rabbit can survive from 4 to 8 days without any irreversible damage, although its weight may drop by 20–30% in less than a week (Cizek, 1961). Although the rabbit is very resistant to hunger and relatively resistant to thirst, any reduction in the water supply, in terms of water requirements, causes a proportional reduction in DM intake, with a consequent drop in performance.

Other environmental factors have also been studied in the domestic rabbit, including the lighting schedule and housing systems. In the absence of light (24-h dark) the feed intake of fattening rabbits is increased, as compared to rabbits submitted to a natural sunlight programme (Lebas, 1977).

As previously mentioned, the type of housing also influences the daily feed intake and feeding pattern of rabbits. For instance, feed intake is affected by the stocking density of rabbits in the cage. An increase

in stocking density could increase the competition for feeders among the animals and reduce feed intake (Aubret and Duperray, 1993). When comparing cage with pen housing, enlarging the cage size for a group (with or without variations in stocking density) allows rabbits to move more, and reduces daily feed intake (Maertens and Van Herck, 2000).

### *1.8 Main problems at weaning*

Rabbits in most farms are weaned between 4 to 5 weeks of age. Because of undeveloped gut immunity system and lack of passive immunity and medium-chain fatty acids (MCFA) protection from doe's milk (Skřivanová and Marounek, 2006; Skřivanová *et al.*, 2008), newly weaned rabbits are susceptible to intestinal infections (mainly caused by *Escherichia coli*, which is the main commensal pathogenic bacteria in rabbit's caecum (Fortun-Lamothe and Boullier, 2007; Carabaño *et al.*, 2008), which can cause high diarrhoea and mortality rate. At the same time, passage from liquid to solid diet, insufficiency of digestive enzyme activity (Gutiérrez *et al.*, 2003; Gomez-Conde *et al.*, 2007) and insufficiency of stomach's acid after weaning can lead to poor digestion and an increased risk of gut disease (Carabaño *et al.*, 2008). The protection of the growing rabbit against enteric infections during the weaning period depends on the synchronization of the transfer from the milk protective mechanism to the gastric protection. Before weaning the digestive system must adapt from a milk-based feeding to an exclusive dependence on solid feed without milk or milk by-products (Carabaño *et al.*, 1998). Young rabbits change from eating a diet rich in animal protein and fat, but poor in carbohydrates, to one based on feed solid with only plant proteins and rich in carbohydrates. Consequently, the digestive system, to reach its full functional capacity, changes from an exclusively endogenous hydrolytic system to one where caecum fermentation becomes important (Pascual, 2001). Thus, the majority of intestinal disease cases (e.g., coliform infections, coccidiosis, Mucoid enteropathy syndrome, Rotavirus-related diarrhoea) in rabbit colonies occur in the weeks immediately following weaning (Orengo *et al.*, 2007). Gastric acidity is considered as the primary defence mechanism against bacterial colonization in the tract (Dinsmore *et al.*, 1997; Boneti *et al.*, 2009) and loss of gastric acidity promotes increased gastric colonization (Donowitz *et al.*, 1986). To tackle these issues several strategies have been adopted over the years. One above all is the use of antibiotics.

### *1.9 The use of antibiotics in animal production*

In animal production, antibiotics have been used both as therapeutic and as growth-promoting agents. Therapeutic usage of antibiotics is typically a high-dose short-term one, the substance is either injected or administered via feed or water. Growth-promoting usage was typically the opposite, i.e., a low-dose long-term administration, usually given in feed. Prophylactic usages, while intentionally therapeutically, can resemble growth-promoting usages, and the latter can have a degree of prophylactic action. Antibiotic growth promoters (AGPs) consistently improved production performances, with most

of the economic benefits being passed to consumers, via lower prices of meat, eggs, and other animal products. AGPs also had secondary advantages: by decreasing feed usage per production unit, AGPs can reduce the amount of land needed for feedstuff production, the imports of feedstuffs from many countries, and the manure volume (manures are a liability in many modern production systems) (Falcão-e-Cunha *et al.*, 2007).

In weaned rabbits, antibiotics were widely used for the prevention of infections caused by digestive disorders and as a growth promoter, altering the gut flora, suppressing bacterial catabolism and reducing bacterial fermentation (Pinheiro *et al.*, 2004).

Increasing worries about food safety led European consumers to oppose the usage of AGPs in animal feeds. Part of the worry with the AGPs had to do with eventual antibiotic residues in meat, milk and eggs. Other consumers, together with a significant part of the medical profession, pointed out that AGPs, being antibiotics administered at low doses and during long time intervals, could lead to microbial resistance in farm animals (Corpet, 1996, Mathew *et al.*, 2007, Hunter *et al.*, 2010). This is a significant criticism and one that is supported by scientific evidence (Wegener, 2006). Since 2006 Europe has banned the use of antibiotics as AGPs. Also, the latest ESVAC report, published in November 2021, showed that the volume of sales of antimicrobials for use in food-producing animals in Europe fell by more than 43% between 2011 and 2020.

Several non-antibiotic substances, commonly described as antibiotic alternatives, helped to ease the transition to a new reality without AGPs. Among the many alternatives, probiotics, prebiotics, symbiotics, enzymes and organic acids were perhaps the most studied and developed. Other alternative products can also be mentioned, such as immune system stimulators and plant or herbal extracts. In common with antibiotics, all these alternative products can act upon the gut microbiota and the gut immune system (Falcão-e-Cunha *et al.*, 2007). In the monogastric field, alternatives to antibiotics were, as expected, mostly studied in pigs and poultry (Thomke and Elwinger, 1998; Doyle, 2001).

### *1.10 Organic acids contribution to reduce the use of antibiotics*

As a group of chemicals, organic acids are considered any organic carboxylic acid of the general structure R-COOH (including fatty acids and amino acids). Organic acids can be classified into three main functional categories: short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA), and tricarboxylic acids (TCA) (Grilli and Piva, 2012). SCFA are carboxylic acids with max 5 carbon atoms and they are produced in the lower intestine of animals by the microbial fermentation of indigestible sugars and amino acids. They are widely distributed in nature as normal constituents of plants or animal tissues and are sometimes found in their sodium, potassium or calcium form.

Thanks to their liquid status, SCFA are mostly used as feed acidifiers, silage inoculants, and preservatives in animal nutrition. SCFA improve intestinal morphology and have a beneficial effect on the intestinal barrier, decreasing intestinal inflammation (Suiryanrayna and Ramana, 2015; Diao, 2019).

MCFA have aliphatic chains with 6 to 12 carbon atoms. These acids can be quickly incorporated in the cells and represent an important energy source (Odle, 1997; Zentek, 2011). Thanks to their high pKa MCFA have a higher antibacterial activity compared to SCFA in the hindgut (Gupta, 2019).
TCA are metabolic intermediates of Krebs cycle, thus involved in energy metabolism. These acids improve gut morphology and barrier function, with a favourable influence on microbiota (Chen, 2018; Li, 2019). Other than these categories, there are a few organic acids like benzoic, sorbic, and lactic acid, that are widely used in food and feed preservation thanks to their antifungal and antimould properties.
When managing organic acids in animal feeding there are several properties to consider, i.e., physical form, flavouring properties like odour and taste, solubility in water and the safety aspect.
There are different organic acids that can act at different levels based on environmental pH: organic acids with higher pKa can be used with the aim of preserving food/feed, while, from a nutritional point of view, a lower pKa means that the acid acts in the stomach of animals. *Table 1.3* shows the common name, chemical name, formula, molecular weight, and first pKa of organic acids that are commonly used as dietary acidifiers for pigs or poultry (Dibner and Buttin, 2002).

*Table 1.3* – List of acids and their properties. (Papatsiros and Christodoulopoulos, 2011).

| **Category** | **Acid** | **Chemical name** | **Formula** | **pKa** |
|---|---|---|---|---|
| SCFA | Formic | Formic acid | CH2O2 | 3.75 |
| SCFA | Acetic | Acetic acid | C2H4O2 | 4.76 |
| SCFA | Propionic | 2-Propanoic acid | C3H6O2 | 4.88 |
| SCFA | Butyric | Butanoic acid | C4H8O2 | 4.82 |
| MCFA | Caproic | Hexanoic acid | C6H12O2 | 5.09 |
| MCFA | Caprylic | Octanoic acid | C8H16O2 | 4.89 |
| MCFA | Capric | Decanoic acid | C10H20O2 | 4.90 |
| MCFA | Lauric | Dodecanoic acid | C12H24O2 | 5.30 |
| TCA | Citric | 2-Hydroxy-1,2,3-Propanetricarboxylic acid | C6H8O7 | 3.13 |
| TCA | Fumaric | 2-Butenedioic acid | C4H4O4 | 3.02 |
| TCA | Malic | Hydroxybutanedioic acid | C4H6O5 | 3.40 |
| Others | Sorbic | 2,4-Hexandienoic acid | C6H8O2 | 4.76 |
| Others | Benzoic | Benzenecarboxylic acid | C7H6O2 | 4.19 |
| Others | Lactic | 2-Hydroxypropanoic acid | C3H6O3 | 3.86 |

### *1.10.1 Antimicrobial properties of organic acids*

Since many years, organic acids are affirmed as powerful antimicrobials. Organic acids owe their success to a broad spectrum of applications, that are based on antibacterial, antiviral, antifungal, and antimould properties (Cochrane *et al.*, 2016; Baltić *et al.*, 2017; Leyva Salas *et al.*, 2017). Not all organic acids have the same antimicrobial activity. Their action against microorganisms depends on the carbon chain length and degree of unsaturation, but overall, the pKa of the acid influences its antimicrobial mechanism of action (Huyghebaert *et al.*, 2011). Organic acids with the highest

antimicrobial activity are short-chain acids (C1-C7) and have a pKa between 3 and 5 (Dibner and Buttin, 2002).

In 1998, Russell and Diez-Gonzalez for the first time proposed the anion model, according to which the inhibitory effect of organic acids is highly related to their undissociated form. Based upon the environmental pH and pKa values, organic acids in their undissociated form can diffuse across the bacterial cell membrane and dissociate inside the cell, releasing H+ ions and decreasing intracellular pH. To overcome the lowering of pH, microorganisms activate proton pumps consuming energy and, at the same time, the anion $RCOO^-$ is toxic to DNA replication, disrupting metabolic functions and increasing osmotic cell pressure (Holyoak *et al.*, 1996; Lambert and Stratford, 1999; Cetin-Karaca, 2011). The combination of these two actions inhibits bacterial replication and growth, leading to bacteriostatic or bactericidal effects. The anion model is generally accepted as the mode of action for all organic acids, but the efficacy of different organic acids can vary mainly on two factors: on one hand, the lipophilic nature of the acid influences the ability to pass through the microorganism wall; on the other hand, upon dissociation inside the cell, different anions can have different inhibitory mechanisms on cellular functions (Hirshfield *et al.*, 2003; van Dam, 2006; Ng and Koh, 2017). As already mentioned, there are many other variables that affect the antimicrobial activity of organic acids besides the pKa. Polar groups, number of double bonds, molecular size, and solubility in non-polar solvents are the four principal chemical and physical features that can predict the inhibitory effect of organic acids, as described by the mathematical model of Principal Components Analysis calculated by Hsiao and Siebert (1999). The spectrum of efficacy of organic acids can vary depending on the nature of the target organism and, in particular, on the complexity and structure of its outer cell wall and/or membrane (Jay *et al.*, 2006). Gram-positive bacteria (i.e., *Clostridium perfringens*, *Enterococcus spp.*, *Streptococcus spp.*) are mainly susceptible to MCFA, while Gram-negative bacteria (i.e., *Escherichia coli*, *Campylobacter jejuni*, *Salmonella spp.*) are more sensitive to SCFA (Giovagnoni *et al.*, 2019; Kovanda *et al.*, 2019). This can be explained by the lipophilic nature of MCFA that allows them to have a stronger antibacterial activity mainly against Gram-positive species, whereas the presence of lipopolysaccharide (LPS) in the gram-negative cell wall confers resistance to these species (Sheu and Freese, 1973). Propionic acid and butyric acid are strong mould inhibitors, while acetic acid is commonly used as an antifungal, also reducing aflatoxins production (Higgins and Brinkhaus, 1999; Pundir and Jain, 2010; El-Kadi, 2015).

*1.10.2 Organic acids in monogastric animals' feeding*

Organic acids have been widely used over the last decades for their positive effect on growth efficiency (eg. increased palatability, feed efficiency, mineral absorption, phytate-P utilization) when supplemented in non-ruminant diets (Partanen and Mroz, 1999; Dibner and Buttin, 2002; Boling *et al.*, 2001). Organic acids with antimicrobial activity are used for the control of intestinal microbial growth (Partanen and Mroz 1999; Davidson, 2001). The antimicrobial activity of organic acids is basically the

same, irrespective of acting in food, feed, or gut lumen (Diebold and Eidelsburger, 2006). Most available information about the use of acidifiers in animal feeding is focused on swine and poultry. First experiments have been carried out in piglets, where organic acids have been administered to compensate for the relatively low gastric production of acid of the young animals, supplemented in diets when subject to early weaning. It was later verified that they could also be advantageous in the later phases of growth, when they could both improve the apparent digestibility of energy and protein, and the absorption and retention of some minerals (Partanen and Mroz, 1999; Diebold and Eidelsburger, 2006). Organic acids were reported as effective growth enhancers throughout the production cycle of pigs, although there is a relatively large variation in responses due to various factors such as type and dose of organic acids used, supplementation duration, type of diet and buffering capacity, hygiene and welfare standards, health status, and age of the animals (Mroz *et al.*, 2006; Tung *et al.*, 2006). The magnitude of growth response was shown to be greater in weaning pigs than in older animals. For example, in a meta-analysis study (Tung and Pettigrew, 2006), the improvements of growth rates were 12.25 and 6.03% for the first 2 weeks or 4 weeks post-weaning respectively, while the enhancement was lower for growing (3.51%) or finishing (2.69%) pigs. Even with a level of variability, all the available literature suggests that organic acids effects on pig growth performance are consistent, thus supporting their use under practical conditions (Tugnoli *et al.*, 2020).

*1.10.3 Effects of organic acids supplementation in rabbits*

The use of organic acids appears interesting, even if scientific data concerning their effect on the microflora population, mucosal immunity and growth performance are few and often contradictory in rabbits due to their peculiar digestive physiology (Falcão *et al.*, 2007). Also, the mode of action of these products on caecal microflora is not completely understood, although it is demonstrated that organic acids play a direct action on performance and health parameters, included mortality, in rabbits (Partanen and Mroz, 1999).

- Organic acids and weight performance:

  According to the scientific literature, the effects of dietary supplementation with organic acids on productive performance of rabbits are inconsistent and incompletely understood (Maertens *et al.*, 2006; Falcão-e-Cunha *et al.*, 2007). However, improvements in daily weight gain have been reported in several studies (Hullar *et al.*, 1996; Scapinello *et al.*, 2001; Michelan *et al.*, 2002), but no positive effects were recorded by others (Hollister et al., 1990). Carraro *et al.* (2005) and Cesari *et al.* (2008) found that dietary inclusion of organic acids had no effect on daily feed intake or growth rate of the fattening rabbits. Contrary to these results, other investigators observed that added dietary organic acids had a positive effect on body weight gain of rabbits (Kamal *et al.*, 2008; Debi *et al.*, 2010; Romero *et al.*, 2011). Adding 15 g fumaric acid/kg diet in study of Scapinello *et al.* (2001) tended to improve rabbit's average daily gain

(ADG), final weight and feed conversion ratio (FCR). The positive effect on growth performance could be due to the reduction of gastric pH by drinking water acidification and subsequent increased gastric enzyme activity and/or bacteriostatic function (Zhu *et al.*, 2014).

- Organic acids and gastrointestinal acidity:

  Gastrointestinal acidity is considered as the primary defence mechanism against bacterial colonization in the tract (Dinsmore *et al.*, 1997; Boneti *et al.*, 2009) and loss of gastric acidity promotes increased gastric colonization (Donowitz *et al.*, 1986). Acidification of the gastrointestinal tract with commercial mixture of organic acids was thought as a way to lower the gastrointestinal pH and compensate for the relatively low gastric production of acid of the animals at weaning. Interesting is the study conducted by Amaefule *et al.* (2011), where is reported that organic acids in diet significantly reduced the pH of rabbit's small and large intestine digesta, but same results were not obtained adding the mixture of organic acid in drinking water. The reason might be that the organic acid in drinking water was absorbed in small intestine (Lanman *et al.*, 1971). In an experiment carried out by Cesari *et al.* (2008), weaned rabbits were divided into four treatment groups characterized by the same pelleted basal diet and by different commercial additives. Some animals received the basal diet without any additive, whereas others were fed with the basal diet supplemented with formic acid and lactic acid. The results showed that the values of caecal pH and total volatile fatty acids (VFA) of rabbits at slaughter were significantly influenced by diets in which treatments were included. Animals fed with treated diets showed a total VFA concentration significantly higher than those fed with other diets. The increase in total VFA and the consequent decrease in caecal pH suggest that fermentation activity was higher where acids have been administered.

- Organic acids and mortality:

  In a study of Skøivanová and Marounek (2002), the inclusion of 0,5% of caprylic acid reduced postweaning mortality, without affecting any other performance trait. In a later trial, Skøivanová and Marounek (2006), testing the medium-chain fatty acids esterified in triglycerides, reached the same results, i.e. a significant reduction in post-weaning mortality, no effect on feed intake, daily gain, or carcass yield. Also, the combination of organic acids with prebiotics (Scapinello *et al.*, 2001) or with probiotics (Michelan *et al.*, 2002) showed significative reduction in mortality, though did not significantly improve performances.

- Organic acids and antimicrobial activity:

  In several studies organic acids had a positive effect on antimicrobial activity, decreasing the rate of *E. coli, Lactobacillus* to total bacteria and markedly lowering the relative proportion of *E. coli* to *Bacteroides–Prevotella* in the caecum (Zhu *et al.,* 2014; Knarreborg *et al.*, 2002). Also, Mehall *et al.* (2001) found that a significant reduction in bacterial translocation and gut colonization occurred within pH 3 and 4 diets, and that a lower pH had a higher effect on

inhibition of bacterial translocation and colonization. In contrast, in other studies testing sodium butyrate (Carraro *et al.*, 2005) and fumaric acid (Scapinello *et al.*, 2001) or formic acid (Skřivanová and Marounek, 2007) no antimicrobial activity was indicated.

## 2. OBJECTIVES

Awareness and knowledge about the use of organic acids in rabbit farming are key in the field to reduce the improve animals' status under commercial conditions and to promote animal health and welfare. Indeed, the use of organic acids appears interesting, even if the scientific publications concerning their effect on the microflora population, mucosal immunity and growth performance are still a few and sometimes contradictory. Also, the mode of action of these products on the gastrointestinal tract is not completely understood. Many studies have been published about the positive effects of organic acids included in pig and poultry farming, but because of the peculiar digestive physiology of the rabbit, it can be hazardous to simply extend the conclusions of studies in other monogastric animals to them.

Thus, the main purpose of the experiment was to evaluate potential organic acids that can be used as acidifiers of weaned rabbit's gastrointestinal tract assessing the effects on performance and microbiota. Specifically, the aim was to evaluate, among several organic acids at different pH, combinations of acids and pH values that best create a barrier against pathogens and thus optimize the health status of animals.

Once the best treatments were selected, the experiment aimed at identifying a simple and replicable process to add the treatments in water and/or feed to improve the intestinal homeostasis, serving as substrate to boost or depress certain bacterial communities as well as provide anti-inflammatory action to restore the mucosal integrity.

The present thesis was developed in the frame on an Erasmus+ (a.y. 2021/22) project lasted 5 months (from March 2022 to July 2022) at the Department of Animal Science of Polytechnic University of Valencia, under the supervision of Professor Pascual Amorós Juan José and Professor Cambra López Maria.

**MATERIAL AND METHODS**

*3.1 Experiment's information and procedure*

The trial run from January 2022 to September 2022 at the Animal Feeding Farm and Laboratory from the Institute of Animal Science and Technology (ICTA), Universitat Politècnica de València (UPV). The research group included Juan José Pascual Amorós (Leader), Enrique Blas Ferrer, María Cambra-López, MªCarmen López Lujan, Eugenio Martínez Paredes, Luís Ródenas Martínez, Chiara Vaggi.
The experimental procedure was approved by UPV's ethical committee and authorised by Conselleria de Agricoltura, Medio Ambiente, Cambio Climático y Desarollo Rural with the code 2022 VSC PEA 0111.
A total of 240 fattening rabbits from the current generation of LP line (resilient line of UPV) were used overall, coming from 21 litters for the first batch and from 19 litters for the second batch. The experimental test was divided into 2 batches of 120 rabbits each. Animals were both sexes. The trial started at 28 d of age and ended at 35 days of age.
Light program was set to 12 hours of light and 12 hours of darkness throughout the study. Environmental conditions during the trial (temperature and ventilation rate) were automatically controlled and adjusted to the age of the rabbits.
A total of 6 organic acids (acetic, formic, propionic, lactic, citric and butyric) at different doses to achieve pH 3, pH 4 and pH 5 were dissolved in water and compared with a control (non-acidified conventional water at around pH 7) (*Table 3.1*). The total number of treatments was of 19 treatments (6 acids x 3 doses + 1 non-acidified control water).

*Table 3.1* – Scheme of the involved acids with pH and identification code.

| **Acid** | **pH** | **Code** |
|---|---|---|
| Control water | ≈7 | CON |
| Acetic acid | 3, 4, 5 | ACET3, ACET4, ACET5 |
| Butyric acid | 3, 4, 5 | BUT3, BUT4, BUT5 |
| Citric acid | 3, 4, 5 | CIT3, CIT4, CIT5 |
| Formic acid | 3, 4, 5 | FOR3, FOR4, FOR5 |
| Lactic acid | 3, 4, 5 | LAC3, LAC4, LAC5 |
| Propionic acid | 3, 4, 5 | PROP3, PROP4, PROP5 |

Organic acids' references were as follows:

- acetic acid (ACET), liquid 99.5%; reference W200603 Sigma-Aldrich. pH=2.5; pKa=ND (4.74 according to literature);
- butyric acid (BUT), liquid 99%; reference B103500 Sigma-Aldrich. pH=2; pKa=4.9;

- citric acid (CIT), solid powder 99%; reference C0759 Sigma-Aldrich. pH=1.7 (100g/L); pKa=3.13;
- formic acid (FOR), liquid 95%; reference F0507 Sigma-Aldrich. pH=2.2; pKa=3.7. Formic acid is not used as pure but is diluted in a solution with 25% of formic acid. Formic acid is too strong to be used easily in its pure form.
- lactic acid (LAC), liquid 85%; reference W261106 Sigma-Aldrich. pH=ND; pKa=ND (3.86 according to literature)
- propionic acid (PROP), liquid 99%; reference 1810 Panreac. pH=2.5; pKA=ND (4.88 according to literature)

Further detailed information about the treatments' preparation can be found in the Annexes section.

At 28 d of age, weaned rabbits were housed in collective cages (50 x 80 x 32 cm), distributing 6 animals per cage and treatment (18 cages for treatments + 1 cage for control water + 1 cage used for the initial sacrifice) having free access to one of the treatments (*Figure 3.1*). Animals were fed *ad libitum* with a commercial diet for growing rabbits until 35 days of age. Water (acidified or not) was also provided *ad libitum* using one bottle drinker per cage, with the capacity of 1.9 litres/bottle (*Figure 3.2*). At weaning, animals were marked with an ear tattoo (*Figure 3.3*). All the animals were weighed at 28, 31 and 35 days of age. Feed and water intake were controlled daily weighing the remaining feed in the feeder and the remaining water in the bottle, considering the eventual spillage. Half of the animals were slaughtered at 31 days of age (3 per treatment) and the other half at 35 days of age (3 per treatment). Additionally, 6 extra animals were slaughtered at 28 days of age to determine basal levels of measures variables. The slaughtering was always carried out from 19:00 to 24:00 to ensure the digestive tract was filled.
A summary of the experimental layout is presented in *Table 3.2*. Farm's cage and treatment arrangement is presented in *Figure 3.4*. The cages' ID goes from number 22 to number 43 (cages 32 and 33 were empty). A summary of the working schedule (for 28, 31 and 35 days of age) is presented in *Table 3.3*.

*Figure 3.1* – Housing conditions at UPV farm.

*Figure 3.2* – Feeders and drinkers labelled for each treatment-pH combination.

*Figure 3.3* – Close up of the ear tattoo. Every animal is marked uniquely.

*Table 3.2* – Summary of experimental layout

| | |
|---|---|
| Total administered treatments | 20 (18 acids + 1 control + 1 initial sacrifice) |
| Total number of batches | 2 |
| Date First batch: 04/04-11/04 | Date Second batch: 09/05-16/05 |
| Total replicate cage/treatment and batch | 1 |
| Total number of animals used | 240 |
| Number of animals/batch | 120 |
| Number of animals/cage | 6 |
| Total number of cages used | 20 (19 treatments + initial sacrifice's cage) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 33 | 34 BUT5 | 35 ACET3 | 36 PROP3 | 37 LAC3 | 38 PROP5 | 39 CIT3 | 40 FOR3 | 41 BUT3 | 42 ACET5 | 43 CIT5 |
| 32 | 31 CIT4 | 30 PROP4 | 29 FOR4 | 28 LAC4 | 27 FOR5 | 26 CON | 25 BUT4 | 24 LAC5 | 23 ACET4 | 22 SACR |

*Figure 3.4* – Farm's cage and treatment arrangement.

*Table 3.3* – Summary of the working schedule of slaughter's days.

| **Working schedule** | **28 day** | **31 day** | **35 day** |
|---|---|---|---|
| **At 9:00** | Weaning, identification and distribution of rabbits in collective cages (6 animals/cage; 1 cage/treatment in each batch) | Feed and water weighing + record of data | Feed and water weighing + record of data |
| | Individual weighing of the animals | Individual weighing of the animals | Individual weighing of the animals |
| | Preparation and distribution of feed and water treatments | Adjustment of water pH and addition of more water if needed | Adjustment of water pH and addition of more water if needed |
| **At 19:00** | Slaughter of 6 animals from the initial sacrifice's cage; measurement and collection of stomach and small intestine pH and sampling stomach content and mucosa for pepsin activity | Slaughter 3 animals/cage; measurement and collection of stomach and small intestine pH and sampling stomach content and mucosa for pepsin activity | Slaughter 3 animals/cage; measurement and collection of stomach and small intestine pH and sampling stomach content and mucosa for pepsin activity |

**Animal recorded traits:**

| |
|---|
| Individual body weight at 28, 31 and 35 days of age |
| Daily feed and water intake per cage at 29, 30, 31, 32, 33, 34 and 35 days of age |
| Daily records of health, illness, culls, mortality, including reason for culls and probable cause of mortality |
| Records of any adverse events (e.g. severe disease outbreak, power cuts, storms, feed/water blockages, etc.) |

**Measurements at slaughtering:** stomach pH (both in fundus and antrum), small intestine pH (at duodenum, jejunum, ileum and caecum), pepsin activity in stomach content at the average pH obtained, and microbial imbalance in the stomach (D-lactate).

A summary of the sampling at slaughtering is presented in *Figure 3.5*.

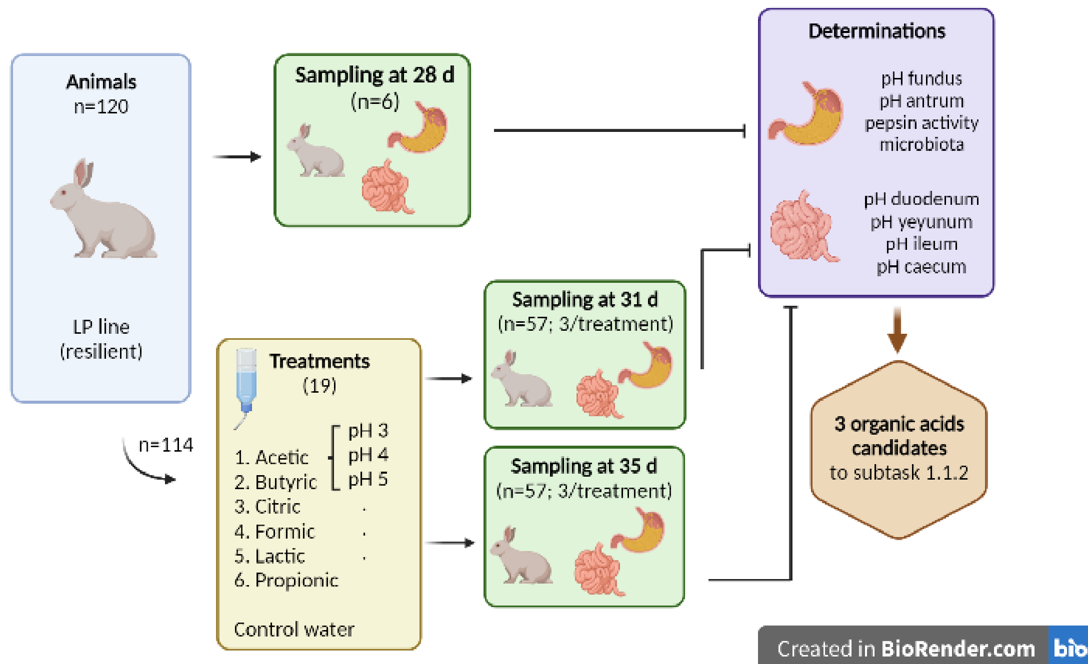


*Figure 3.5* - Experimental scheme of sampling activity.

## *3.2 Feed, water and diet analysis*

Animals were fed commercial non-medicated fattening rabbit pelleted feed (CUNIVITA Sd G GR) manufactured by NANTA, including alfalfa meal, wheat bran, sunflower meal, wheat straw, barley, sugar beet pulp, ground and dried mixture of bakery and biscuit by-products, soybean oil, beet molasses, sodium chloride, yeasts and parts thereof, corn gluten flour, dried seaweed, calcium carbonate. Diet was pelleted with pellet size from 2.5-3.5 mm x 5.0-8.0 mm.

The diet was formulated to meet nutrient requirements for fattening rabbits *(De Blas and Mateos, 2010)* and its chemical composition was crude protein: 14.5%, fat 4.0%, fiber 19.7%, ashes 7.5%, calcium 0.80%, phosphorus 0.45%, sodium 0.28%.

The weeks before the first trial, dosing, dissolution, and pH stability tests were carried out with the 6 acidifiers to be evaluated in the laboratory. The objective of these preliminary tests was to determine the greater or lesser difficulty of dissolving each acidifier, the amount of product necessary to reach the target pH and the change in pH that occurs over time (in a period of 24 hours: 0, 3, 6, 12 and 24 hours after preparation).

The amount of drinking water included in each drinker was controlled initially as well as the pH of the treatment. When necessary, more adjusted drinking water was added to ensure *ad libitum* access to water. All additions were registered to determine the daily water consumption in each cage.
As for chemical analysis of diets, a 500-g representative feed sample was taken during the trial and grounded with a hammer mill with a 1 mm sieve. Sample were analysed for dry matter (DM), ashes, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), ether extract (EE) and starch by UPV.
Methods of the AOAC (934.01, 2000), ISO (1999) and EGRAN (2001) were used for dry matter. Methods of the AOAC (942.05, 2000) and ISO (1978) were used for ashes. methods of AOAC (920.39, 2000) were used for ether extract (crude fat) with acid-hydrolysis of samples prior to the extraction. Methods of AOAC (990.03, 992.23, 2002), UNE-EN ISO 16634-1 (2009), Leco Manual CN628 and Simonne *et al.* (1994) were used for crude protein (Dumas Method). The NDF, ADF and ADL fractions were analysed sequentially according to Mertens *et al.* (2002), AOAC (973.18, 2000) and Robertson and Van Soest (1981), respectively, with a thermos-stable α-amylase pre-treatment and expressed exclusive of residual ash, by using a nylon filter bag system (Ankom, Macedon, NY, USA).
The laboratory analysis procedures written below follow the guidelines of the *"Manual de prácticas de laboratorio en nutrición animal"* (Salvador *et al*., 2016).
Further detailed information about the analysis of diets can be found in the Annexes section.

### *3.3 Feed analysis results*

The same diet was used for all animals from both Batch 1 and Batch 2 and provided from days 28 to days 35 of age. It was a commercial non-medicated fattening rabbit pelleted feed suitable for weaning rabbits. The lab analysis (Table 3.4) showed that the chemical composition of the diet was in accordance with feed label (data not shown) and within the nutrient requirements recommended for fattening rabbits (De Blas and Mateos, 2010) except for the amount of fiber, which was higher than recommendations, i.e. NDF 36-39% DM and ADF 20-22% DM.

*Table 3.4* – Feed composition results (% dry matter)

| **Dry matter (%)** | **Ashes (%DM)** | **Crude protein (%DM)** | **Starch (%DM)** | **Ether extract (%DM)** | **NDF (%DM)** | **ADF (%DM)** | **ADL (%DM)** |
|---|---|---|---|---|---|---|---|
| 90.38 | 8.06 | 16.45 | 17.41 | 4.14 | 45.67 | 22.17 | 3.77 |

NDF: neutral derteng fiber; AFS: acid detergent fibre, ADL: acid detergent lignin.

Since it is necessary to offer young rabbits a diet suited to their digestive capabilities through age, the best feeding strategy around weaning seems to provide a high-fibre and low-starch diet to the young rabbits, as this improves the health and the fastest growth of fattening rabbits (Butcher *et al.*, 1983).

Consistently with the finding of Butcher *et al.* (1983), in the current experiment a diet higher in fiber and lower in starch was fed to weaned rabbits.

*3.4 Slaughtering*

Immediately after slaughtering, the abdominal cavity was opened, and the entire gastrointestinal tract was removed to analyse the stomach pH and small intestine pH. The gastric mucosa was also sampled and stored for the analyses of the pepsin activity.

Stomach content from fundus and antrum was separated in 2 beakers and homogenized before pH determination with a pH meter (Vio XS, XS Instrument) using a 2 PORE Steel T electrode (XS Sensor). The full content of duodenum, jejunum, ileum and caecum was collected in 4 tubes of 10 ml and homogenized before pH determination with a pH meter (Vio XS, XS Instrument) using a MICRO electrode (XS Sensor).

After fundus and antrum pH determination, both contents were joined and homogenized, and a representative sample was taken. The sample (10 g) was frozen in liquid nitrogen and stored at -80°C for determination of pepsin activity. After stomach was washed in ice normal saline, the mucosa was gently scraped off with a microscope slide and mucosa sample were stored at -20°C until further analysis.

*3.5 Statistical analysis*

Data were submitted to ANOVA to evaluate the effect of the experimental treatments, the inclusion of acids, the day of sampling/recording as well as the effect of the batch. Contrasts of group means among each of the experimental treatments against negative control (basal diet) were done using Dunnett test. Significant differences were declared at $P \leq 0.05$. SAS Software 9.3 is used as the statistical software/program. Any outliers removed prior to statistical analysis has been indicated with an explanation note.

## RESULTS AND DISCUSSION

### *4.1 Rabbits performance*

The data collected during the trial were about live body weight, water and feed intake (AWI and AFI, respectively), and the pH of stomach and intestine at slaughtering. All data are related and grouped by the specific treatment and/or general acid administered. Data from both Batch 1 and Batch 2 have been merged and analysed together. Similarities and differences among all acids, and between each treatment or acid and the control water (CON) have been highlighted. *Table 4.1* shows to type of analysis. The effects the 19 treatments (which appears with non-bold letters), and the effect of the acid used (which appears with bold letters). In addition, the table also shows the lineal effect of the pH (trait change per pH unit).

All treatments were compared with the control water treatment (marked as CON TREAT on the table) to assess which treatments present a statistical difference from the control treatment according to the performance parameters considered.

*Table 4.1* – effect of the experimental treatments on performance of rabbits from 28 to 35 days of age

| Treatment | BW28 (g) | BW31 (g) | BW35 (g) | ADG28-31 (g) | ADG31-35 (g) | ADG TOTAL (g) | ADFI (g) | ADWI (g) |
|---|---|---|---|---|---|---|---|---|
| CON TREAT | 527.4 | 600.8 | 760.1 | 24.17 | 36.33 | 32.29 | 61.40 | 149.12 |
| **CON ACID** | **530.1**$^{a}$ | **610.3**$^{ab}$ | **774.2**$^{a}$ | **26.67**$^{ab}$ | **36.88**$^{ab}$ | **33.45**$^{a}$ | **61.40**$^{a}$ | **149.1**$^{a}$ |
| **ACET** | **533.2**$^{a}$ | **600.5**$^{a}$ | **728.0**$^{ab}$ | **22.42**$^{ab}$ | **35.00**$^{ab}$ | **28.21**$^{bc}$ | **56.00**$^{b}$ | **117.0**$^{bc}$ |
| ACET 3 | 528.5 | 541.9$^{*}$ | 692.8 | 4.59$^{*}$ | 40.9 | 25.2 | 44.93$^{*}$ | 71.41$^{*}$ |
| ACET 4 | 534.3 | 615.9 | 714.7 | 27.4 | 30.8 | 28.1 | 58.20 | 137.9 |
| ACET 5 | 532.3 | 629.6 | 800.1 | 32.6 | 42.7 | 37.0 | 64.89 | 141.7 |
| **BUT** | **532.8**$^{a}$ | **560.7**$^{b}$ | **686.1**$^{b}$ | **9.31**$^{c}$ | **34.93**$^{ab}$ | **22.94**$^{c}$ | **48.54**$^{c}$ | **107.2**$^{c}$ |
| BUT 3 | 530.0 | 467.8$^{*}$ | 540.4$^{*}$ | -20.8$^{*}$ | 28.8 | 6.04$^{*}$ | 29.94$^{*}$ | 43.32$^{*}$ |
| BUT 4 | 530.1 | 607.8 | 747.5 | 25.9 | 39.1 | 32.5 | 58.63 | 135.8 |
| BUT 5 | 540.9 | 606.7 | 768.6 | 22.0 | 34.7 | 28.9 | 57.05 | 142.4 |
| **CIT** | **535.9**$^{a}$ | **625.3**$^{a}$ | **770.6**$^{a}$ | **26.67**$^{a}$ | **35.28**$^{ab}$ | **33.45**$^{ab}$ | **61.83**$^{a}$ | **143.0**$^{a}$ |
| CIT 3 | 533.1 | 624.1 | 768.7 | 30.3 | 34.0 | 33.1 | 63.00 | 139.1 |
| CIT 4 | 533.6 | 603.6 | 761.6 | 23.2 | 37.9 | 32.1 | 56.66 | 109.6 |
| CIT 5 | 535.4 | 633.4 | 794.9 | 32.7 | 42.2 | 39.7 | 65.83 | 180.3 |
| **FOR** | **530.1**$^{a}$ | **618.3**$^{a}$ | **769.7**$^{a}$ | **29.40**$^{a}$ | **37.92**$^{a}$ | **35.60**$^{a}$ | **62.20**$^{a}$ | **124.2**$^{b}$ |
| FOR 3 | 534.0 | 621.5 | 800.1 | 29.1 | 38.3 | 36.1 | 62.50 | 111.2 |
| FOR 4 | 525.0 | 610.2 | 756.3 | 28.6 | 40.8 | 36.9 | 61.13 | 124.4 |
| FOR 5 | 535.4 | 623.0 | 753.4 | 29.3 | 38.2 | 35.5 | 62.99 | 137.1 |
| **LAC** | **526.4**$^{a}$ | **604.2**$^{a}$ | **701.7**$^{ab}$ | **25.93**$^{ab}$ | **29.11**$^{b}$ | **27.30**$^{bc}$ | **54.28**$^{b}$ | **157.4**$^{a}$ |
| LAC 3 | 519.4 | 591.9 | 726.0 | 24.7 | 26.7 | 28.0 | 59.10 | 179.7 |
| LAC 4 | 527.1 | 606.3 | 697.5 | 26.6 | 25.3$^{*}$ | 25.5 | 49.91$^{*}$ | 148.6 |
| LAC 5 | 527.6 | 602.9 | 669.1 | 25.4 | 31.4 | 26.0 | 53.85 | 143.7 |
| **PROP** | **533.2**$^{a}$ | **589.6**$^{ab}$ | **682.5**$^{b}$ | **18.80**$^{b}$ | **29.94**$^{b}$ | **23.96**$^{c}$ | **49.60**$^{c}$ | **106.2**$^{c}$ |
| PROP 3 | 521.2 | 493.8$^{*}$ | 595.0$^{*}$ | -9.03$^{*}$ | 26.2 | 9.75$^{*}$ | 33.09$^{*}$ | 43.24$^{*}$ |
| PROP 4 | 532.5 | 633.3 | 770.8 | 33.6 | 34.4 | 34.9 | 60.05 | 117.0 |
| PROP 5 | 533.8 | 622.2 | 728.6 | 29.6 | 29.2 | 28.8 | 55.66 | 158.2 |
| SEM TREAT | 16.07 | 20.77 | 38.88 | 3.92 | 4.35 | 4.07 | 1.877 | 8.570 |
| **SEM ACID** | **10.28** | **14.56** | **24.33** | **3.10** | **2.65** | **2.66** | **1.82** | **7.74** |
| **p-value ACID** | **0.9957** | **0.0192** | **0.0093** | **<0.0001** | **0.1214** | **0.0016** | **<0.0001** | **<0.0001** |
| pH ESTIMATE | +0.08 ±4.69 | +27.71 ±6.39$^{*}$ | +25.59 ±10.83$^{*}$ | +9.21 ±1.31$^{***}$ | +0.02 ±1.22 | +3.99 ±1.18$^{**}$ | +5.64 ±0.76$^{***}$ | +26.3 ±3.103$^{*}$ |
| p-value TREAT | 1.000 | <0.0001 | <0.0001 | <0.0001 | 0.0061 | <0.0001 | <0.0001 | <0.0001 |
| p-value day | - | - | - | - | - | - | <0.0001 | <0.0001 |
| p-value batch | - | - | - | - | - | - | <0.0052 | <0.0001 |

CON: control; ACET: acetic acid; BUT: butyric acid; CIT: citric acid; FOR: formic acid; LAC: lactic acid, PROP: propionic acid
3: pH 3; 4: pH 4; 5: pH 5
Means with $^{*}$ differ significantly from CON at P<0.05
$^{a,b,c}$Means with different letters within a column differ significantly at P<0.05
pH linear effect: degree of change in the variable varying one unit of pH. *P<0.05; **P<0.01; ***P<0.001
SEM: standard error of the mean
The P-value threshold set for SAS analysis is of 0.05 points.

- Treatment value ≤ 0.05 → the treatment is significantly different from the CON TREAT (marked with an asterisk on the table)
- Treatment value > 0.05 → the treatment isn't significantly different from the CON TREAT

Water intake of the cage was recorded daily from day 28 to day 35 of age and ADWI was obtained for each animal (*Figure 4.1*). The ADWI was on average equal to 127.0 g/day. Rabbits from treatments ACET3, PROP3 and BUT3 showed significantly lower ADWI compared with the control (from -77 to -106 g ADWI) while the other experimental groups did not differ from CON (P>0.05). When analyses were performed grouped by ACID, independent from the pH, ADWI was significantly lower in all treatments compared with the control group where acid was added to the water, except for CIT and LAC treatments.

The addition of acids to the water reduced water pH but this reduction did not statistically increase ADWI. In fact, the pH ESTIMATE value for ADWI equalled +26 g, indicating that ADWI was favoured in treatments were the pH was higher.

*Figure 4.1* represents the evolution of daily water intake of rabbits according to the acid used. In all rabbits treated with acids, water intake increased with age (P<0.05) due to the growth animals, showing a peak between the fourth and the fifth day. The intake of BUT and PROP was always lower than other treatments and the control group. Although the water intake of ACET was low during the first two days, it markedly increased from day 3 to the end of the experiment (indicating animal's adaptation to the water treatment), reaching the same values of the other treatments and the control group.

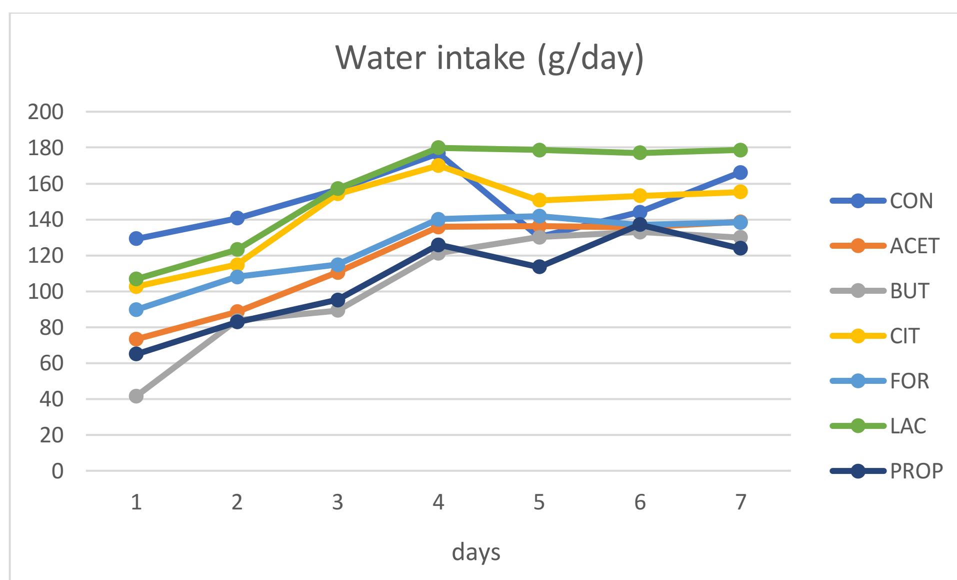


*Figure 4.1* - Average daily water intake grouped by acid

Overall, these data show animals from the control group showed significantly higher performance compared to the tested treatments. The addition of acids to the water during the first 7-day administration negatively affected ADG and ADFI in all treatments except for CIT and FOR treatments which showed similar values in terms of performance to the control group. Indeed, no acid demonstrated to diverge relevantly in positive from the CON. On the contrary, there were some acids that impaired growth, suggesting that there could be some acids or treatments not recommended for rabbits (i.e. ACET, BUT, LAC and PROP).

In general, the reduction of water pH reduced the performances. Using water or acids at pH 4 or 5 provided comparable results to the control group, while using pH 3 acids impaired the growth and feeding behaviour of rabbits, particularly in strongly odorous acids such as ACET3, BUT3 and PROP3. The reason why rabbits ingested a lower amount of these treatments (compared to the other treatments) relies on the organoleptic properties, not so palatable, of these products, enhanced also by the low pH of 3 when compared with the control treatment.

Feed intake was on average equal to 55.7 g/day. In accordance with the average growth performance, rabbits from PROP and BUT acids showed significantly lower ADFI compared with the control group (from -16 to -31 g ADFI), being the rest similar ($P<0.05$) to CON. Animals from treatment LAC also showed significantly lower ADFI compared with the control group (-12 g ADFI). Grouped data for CIT and FOR treatments showed highest results, compared with the control group (*Figure 4.2*).

The addition of acids to the water reduced water pH but this reduction did not statistically increase ADFI. In fact, the pH ESTIMATE value for ADFI equalled +5 g, indicating the increase in pH favoured ADFI.

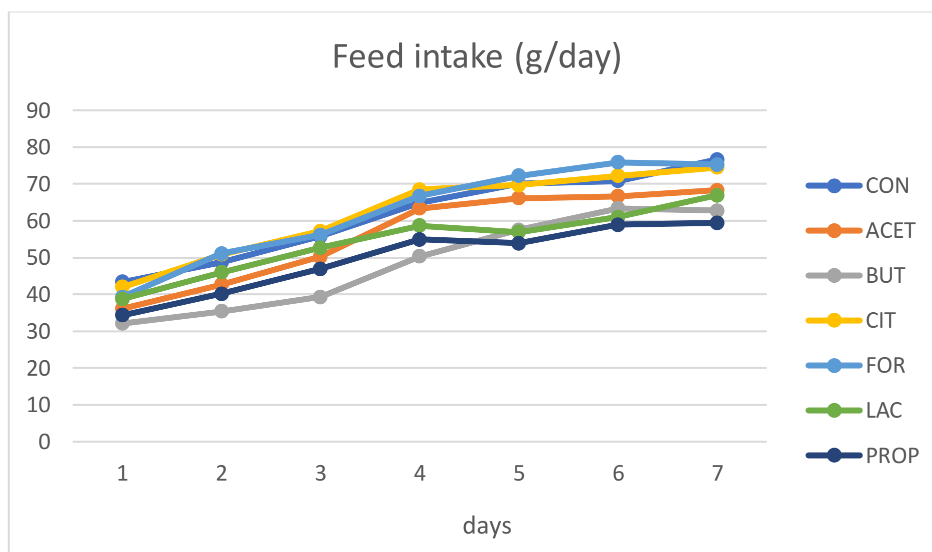


*Figure 4.2* - Average daily feed intake grouped by acid

The individual live body weight (BW) of all animals was recorded at 28, 31 and 35 days of age. At weaning, average BW was equal to 530.6 g without differences in BW amongst treatment as due. At the end of the experiment, (day 35), animals weighed 728.7 g on average. However, there were differences in BW amongst treatments.

Animals from treatments ACET3 (day 31), PROP3 and BUT3 (days 31 and 35) showed a significantly lower BW and ADG compared with the animals from the control group, being the others similar ($P<0.05$) to CON. Particularly, these three acids showed a strong smell: ACET vinegar-like, PROP pungent and rancid, and BUT strongly unpleasant similar to rancid butter. This issue could have affected animal's drinking behaviour, resulting in animal's disliking the taste and odour of these acids at high concentrations, negatively affecting feed intake and ADG. Consequently, ADG during the first period

(28 to 31 days) was lower in animals from treatments BUT3 and PROP3, improving thereafter (from 31 to 35 days) showing a certain adaptation to the taste and smell of the water treatments. This adaptation, however, was insufficient to reach the BW or ADG levels of the animals from the control group or rest of treatments.

When analyses were performed grouping data by type of acid (ACID), independent from the pH, there were no differences in BW at 31 days with any of the acid treatments compared with the control. At 35 days of age, however, PROP and BUT groups showed significantly lower BW compared with the control. The ADG from 28 to 31 days was also significantly lower in BUT treatment compared with the control. No differences were observed in ADG from 31 to 35 days compared with the control when grouping data by acid. As regards total ADG, it was significantly lower in all treatments where acid was added to the water, except for CIT and FOR treatments compared with the control group.

The addition of acids to the water reduced water pH, but this reduction did not statistically increase weight gain. In fact, the pH ESTIMATE value for BW varied from +28 g to +26 g, indicating the increase in pH favoured BW. The same for ADG, where pH ESTIMATE value varied from +0 g to + 9 g, indicating the increase in pH favoured ADG, as well.

*4.2 Changes in gut pH*

*Table 4.2* shows the effects the 19 treatments (which appears with non-bold letters), and the effect of the acid used (which appears with bold letters) on gut pH. Data from both Batch 1 and Batch 2 have been merged and analysed together. In addition, the table also shows the lineal effect of the pH (trait change per pH unit).

The pH varied along the digestive tract. Average values for pH among treatments were equal to 3.84 in the fundus, 1.52 in the antrum, 7.17 in the duodenum, 7.49 in the jejunum, 7.62 in the ileum and 5.67 in the cecum. We observed a statistically significant effect of batch on antrum pH and of the sampling day on ileum and cecum pH.

Firstly, as regards gastric pH, there were generally no differences in the pH values in any of the tested water treatments compared with the control group. This indicated a limited capacity of acids to reduce stomach pH (fundus and antrum). Instead, some treatments (LAC4 in fundus; and CIT4 and CIT5 in antrum) showed a significant increase in pH compared with CON.

When analyses were performed grouped by ACID, independent from the pH, the pH in the fundus was significantly higher in ACET, CIT and LAC treatments compared with the control group. The pH in the antrum was significantly higher in CIT grouped treatments compared with the control group.

The pH ESTIMATE value for pH fundus equalled +0.1 and +0.02 in the antrum. This indicated the reduction in pH reduced the pH content in the fundus and antrum. However, this estimate was not statistically significant.

Moreover, higher pH values were found in the stomach right after suckling/feeding, suggesting that the amount of milk/feed intake and time elapsed from the meal could play an important role on gastric pH values because of a hypothetical basic pH of ingested content and a buffering effect.

Secondly, as regards pH in the small intestine, there were no differences in the pH values in any of the tested water treatments compared with the control group in the duodenum, jejunum and ileum. This indicated a limited capacity of acids to reduce pH in these gut segments. Instead, BUT3 in the duodenum, and ACET3, FOR4 and PROP3 in the jejunum showed a significant increase in pH compared with CON. In the ileum, PROP3 also significantly increased pH compared with the control group.

When analyses were performed grouped by ACID, independent from the pH, the pH in the duodenum was significantly lower in CIT grouped treatments and in ACET and LAC in the ileum.

The pH ESTIMATE value for pH in the duodenum equalled -0.06; -0.03 in the jejunum and -0.03 in the ileum. This indicated the reduction in pH increased ($P>0.05$) the pH content in these gut segments using all data.

Finally, as regards pH in the cecum, there were no differences in the pH values in any of the tested water treatments compared with the control group. Again, this indicated a limited capacity of acids to reduce pH in the cecum. Instead, BUT3 showed a significant increase in pH compared with CON.

However, when analyses were performed grouped by ACID, independent from the pH, the pH in the cecum was significantly lower in ACET, CIT, FOR, LAC and PROP. The pH ESTIMATE value for pH in the cecum equalled -0.06. This indicated the reduction in pH increased ($P>0.05$) the pH content in the cecum using all data.

Overall, results of gut pH indicated that under the conditions tested in this experiment, conducted under unrestricted feeding conditions, the capacity of acids to lower pH in the proximal segments of the gut is limited. This result could have been probably affected by the high buffering capacity of feed that could be masking the effect of the acids to lower pH and thus attenuating their potential. However, results show that when acids were added to water, in some cases, particularly with strong acids such as ACET, FOR, LAC and PROP, but also in CIT, their potential to lower pH was observed in the distal segments of the gut, specifically in the cecum. These results indicate the ability of some acids (independent of the pH) to maintain their properties beyond the stomach, exercising their antimicrobial effects at different sites.

*Table 4.2* – Gastrointestinal pH values correlated with treatments/acid: output values

| **Treatment** | **Fundus** | **Antrum** | **Duodenum** | **Jejunum** | **Ileum** | **Cecum** |
|---|---|---|---|---|---|---|
| CON TREAT | 3.35 | 1.46 | 7.16 | 7.44 | 7.60 | 5.63 |
| **CON ACID** | **2.94**$^{cd}$ | **1.37**$^{bc}$ | **7.40**$^{a}$ | **7.57**$^{a}$ | **7.73**$^{a}$ | **5.84**$^{a}$ |
| **ACET** | **3.94**$^{ac}$ | **1.51**$^{bc}$ | **7.18**$^{ad}$ | **7.46**$^{a}$ | **7.59**$^{bcde}$ | **5.66**$^{b}$ |
| ACET 3 | 3.79 | 1.44 | 7.33 | 7.56$^{*}$ | 7.71 | 5.70 |
| ACET 4 | 4.09 | 1.55 | 7.12 | 7.41 | 7.51 | 5.70 |
| ACET 5 | 3.80 | 1.53 | 7.12 | 7.43 | 7.56 | 5.61 |
| **BUT** | **3.61**$^{bcd}$ | **1.49**$^{bc}$ | **7.20**$^{ab}$ | **7.49**$^{a}$ | **7.64**$^{ac}$ | **5.73**$^{a}$ |
| BUT3 | 2.95 | 1.47 | 7.45$^{*}$ | 7.50 | 7.71 | 5.88$^{*}$ |
| BUT4 | 3.76 | 1.53 | 7.13 | 7.52 | 7.57 | 5.66 |
| BUT5 | 4.13 | 1.47 | 7.02 | 7.49 | 7.63 | 5.68 |
| **CIT** | **3.99**$^{ab}$ | **1.60**$^{a}$ | **7.13**$^{bcdef}$ | **7.51**$^{a}$ | **7.60**$^{ad}$ | **5.62**$^{b}$ |
| CIT3 | 4.21 | 1.49 | 7.11 | 7.53 | 7.62 | 5.70 |
| CIT4 | 4.03 | 1.62$^{*}$ | 7.24 | 7.50 | 7.65 | 5.65 |
| CIT5 | 3.98 | 1.66$^{*}$ | 7.04 | 7.48 | 7.55 | 5.57 |
| **FOR** | **3.83**$^{ad}$ | **1.53**$^{ac}$ | **7.16**$^{ae}$ | **7.47**$^{a}$ | **7.60**$^{ae}$ | **5.62**$^{b}$ |
| FOR3 | 4.24 | 1.57 | 7.18 | 7.46 | 7.61 | 5.66 |
| FOR4 | 4.10 | 1.56 | 7.07 | 7.53$^{*}$ | 7.62 | 5.64 |
| FOR5 | 3.27 | 1.45 | 7.19 | 7.41 | 7.58 | 5.61 |
| **LAC** | **4.14**$^{a}$ | **1.54**$^{ab}$ | **7.15**$^{af}$ | **7.47**$^{a}$ | **7.59**$^{cde}$ | **5.65**$^{b}$ |
| LAC3 | 3.78 | 1.52 | 7.22 | 7.53 | 7.64 | 5.74 |
| LAC4 | 4.94$^{*}$ | 1.56 | 7.06 | 7.44 | 7.53 | 5.62 |
| LAC5 | 3.81 | 1.51 | 7.18 | 7.46 | 7.60 | 5.63 |
| **PROP** | **3.58**$^{bcd}$ | **1.49**$^{bc}$ | **7.20**$^{ac}$ | **7.50**$^{a}$ | **7.66**$^{ab}$ | **5.64**$^{b}$ |
| PROP3 | 2.76 | 1.38 | 7.40 | 7.61$^{*}$ | 7.76$^{*}$ | 5.77$^{*}$ |
| PROP4 | 4.02 | 1.51 | 6.97 | 7.47 | 7.61 | 5.59 |
| PROP 5 | 3.98 | 1.56 | 7.26 | 7.46 | 7.66 | 5.61 |
| SEM TREAT | 0.3593 | 0.0640 | 0.0962 | 0.0443 | 0.0499 | 0.0485 |
| **p-value ACID** | **0.0942** | **0.0537** | **0.5169** | **0.3759** | **0.1488** | **0.0022** |
| pH ESTIMATE | +0.098 ±0.102 | +0.022 ±0.017 | -0.058 ±0.027$^{*}$ | -0.034 ±0.012$^{*}$ | -0.028 ±0.014$^{*}$ | -0.059 ±0.014$^{*}$ |
| p-value TREAT | 0.0002 | 0.0967 | 0.0057 | 0.0427 | 0.0633 | 0.0006 |
| p-value day | 0.117 | 0.470 | 0.411 | 0.225 | 0.0002 | 0.0074 |
| p-value batch | 0.228 | 0.0005 | 0.300 | 0.292 | 0.380 | 0.671 |

CON: control; ACET: acetic acid; BUT: butyric acid; CIT: citric acid; FOR: formic acid; LAC: lactic acid, PROP: propionic acid

3: pH 3; 4: pH 4; 5: pH 5

Means with $^{*}$ differ significantly from CON at $P<0.05$

$^{a,b,c}$Means with different letters within a column differ significantly at $P<0.05$

pH linear effect: degree of change in the variable varying one unit of pH. *P<0.05; **P<0.01; ***P<0.001

SEM: standard error of the mean

The P-value threshold set for SAS analysis is of 0.05 points.

- Treatment value ≤ 0.05 → the treatment is significantly different from the CON TREAT (marked with an asterisk on the table)
- Treatment value > 0.05 → the treatment isn't significantly different from the CON TREAT

Additionally, the graph in *Figure 4.3* illustrates the interaction between body weight at slaughter and pH of stomachs and intestine from animals. The values collected show that body weight at slaughter does not affect the pH score of animals' innards. Each intestinal segment maintains its pH value steady regardless of the body weight at slaughter of rabbits.

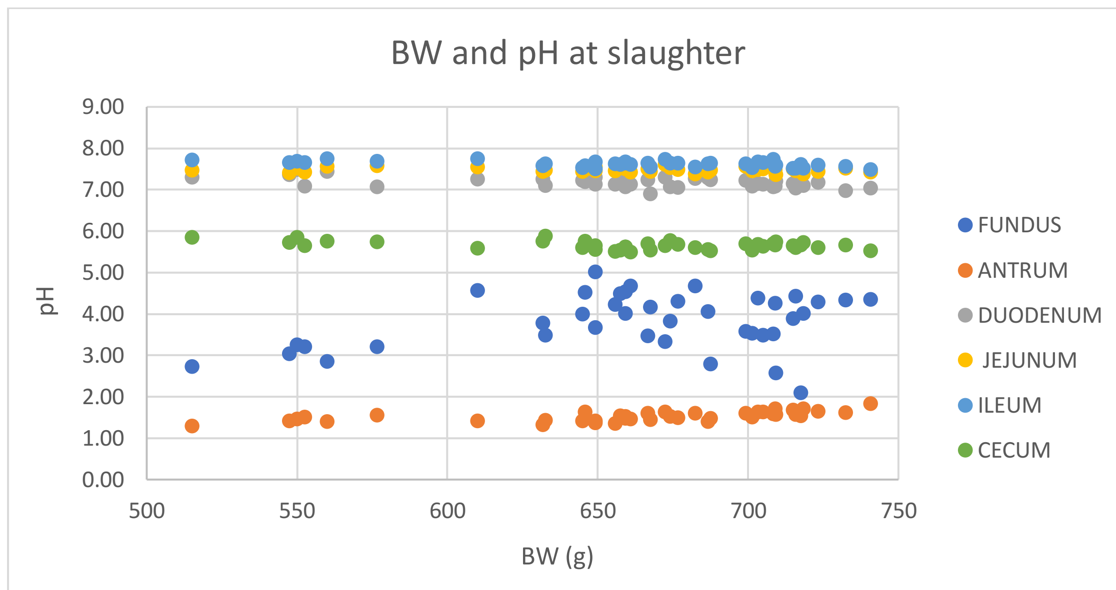


*Figure 4.3* – Animals' body weight at slaughter and pH scores related together.

*4.3 Discussion*

Our results gave a first insights about the experiments that can be carried out to assess the effectiveness of some organic acids in rabbit farming.

The low number of acids/treatments that statistically overcome the control water in the expected way (mainly by lowering the pH in the gut) suggested a limited potential of acids in lowering down the gut pH.

The results obtained within the present thesis, indeed, did not fully confirm the initial hypothesis. This could be explained by several reasons:

1. acid-related factors: behaviour and properties of acids themselves (type and dose of acid, different pKa, and duration of the supplementation period);
2. animal-related factors: animal's age, weight, health status, physiological development, etc;
3. feed-related factors: feed intake and buffering capacity of feed and endogenous intestinal secretions.

Regarding the type and dose of acids, the organic acids chosen for the experiments were almost all short-chain fatty acids administered in a liquid form mixed with water. These acids are recommended in monogastric species and authorised as animal feed additives (preservatives, mainly) by European Food Safety Authority (EFSA). Quantity of acid used differed amongst all treatments (see *Table 6.2* on Annexes). Treatments with pH 5 needed a very lower dosage of acid if compared to treatments at pH

3. Treatments with higher dose of acid could be more effective, but the increase of acids makes water also less palatable and rabbits are deterred from drinking. Results showed, indeed, lower performance results in cages where treatments with higher acid supplementation were administered.

As for the supplementation duration, treatments were given for 7 days, from day 28 to day 35 of age of animals. Previous works in rabbits, although scarce, have tested acidifiers (organic acids either in feed or water) during the whole fattening period (usually until 56-80 days of age) (Cesari *et al.*, 2008; Romero *et al.*, 2011; Zhu *et al.*, 2014) achieving positive results in performance, regulating gastric pH, improving the microbial ecosystem and gut health. These studies, however, both used a blend of acids composed of formic acid, acetic acid and ammonium formate salt (Zhu *et al.*, 2014); encapsulated formic and citric acids (Romero *et al.*, 2011), or a blend of formic and lactic acid (Cesari *et al.*, 2008). The combination of acids and their salts could have synergic effects in rabbits performance and gut traits. Supplementing the acids for a longer time could have led to different results.

Moreover, as regards acid-associated factors, the efficiency of a given acid depends on its pKa, the pH value at which the acid is half dissociated. The pKa is used to indicate the strength of an acid. A lower pKa value indicates a stronger acid, that is, the lower value indicates the acid more fully dissociates in water. The pKa values of the organic acids implemented in our experiment are listed on *Table 1.3*.

Among the chosen acids from our experiment, propionic acid has the highest pKa (4.88), while citric acid shows the lowest value (3.13). Higher pKa acids tend to be more effective (Falcão-e-Cunha *et al.*, 2007). On the other hand, the antimicrobial effectiveness of organic acids tends to increase both with their chain length and their degree of unsaturation (as reviewed by Partanen and Mroz, 1999).

As regards feed-associated factors, buffering capacity is defined as the ability of a material to resist changes in pH after addition of acid or alkali. It is important to consider feed buffering capacity during gastric digestion as it impacts the intragastric pH and gastric secretion rate. The more the feed in the stomach and the less the acid works. Thus the feed intake is correlated to the buffering capacity of feed. Where rabbits eat most it's more likely to have a limited potential of treatments, even though the buffering capacity of feed is a momentaneous condition related to feed digestion. Buffering capacity of endogenous intestinal secretions need to be considered too, since the vermiform appendix can secrete bicarbonate that buffers the caecal acids and, thus, it could be an explanation of why in the intestinal tract pH is maintained tendentially stable (Zhu *et al.*, 2014).

As described in de Blas and Wiseman (2002), soft faeces and saliva, together with the buffering capacity of the diet can prevent immediate acidification. When the rabbit practices caecotrophy, the pH of the fundus is almost neutral, whereas the pH of the antrum always remains acidic (<3) (Gidenne and Fortun-Lamothe, 2002). Additionally, rabbits after weaning suffer physiological hypochlorhydria and thus gastric pH can be higher than 4 in 3 to 4-week-old rabbits (Gidenne and Fortun-Lamothe, 2002). Gastric pH in suckling rabbits is high because it is necessary for bacterial colonization of the intestinal tract (Vennen and Mitchell, 2009). The introduction of solid feed represents a new step in the dynamic construction of the gut microbiota (Paës *et al.*, 2022) and thus gut pH barrier could be reduced naturally.

Around weaning, additionally, stomach pH can also physiologically rise because there is a decrease in lactic acid production due to the reduced milk intake and the change in feed intake patterns consuming larger quantities of feed that could attenuate organic acid effect in the stomach.

Therefore, the stomach of rabbits at early weaning is physiologically maintained at a high pH (often over 5), because the gastro-intestinal tract of rabbits is not fully developed and this causes a poor endogenous HCl secretion, lack of lactic acid from lactose fermentation, and intake of large meals at infrequent intervals (Suiryanrayna and Ramana, 2015). Beside these physiological aspects, gastric pH of weaning rabbits is kept high also by the high buffering capacity of a diet still based on milk products. Consequently, it is not easy to lower down the stomach pH with the inclusion of organic acids (Tugnoli *et al.*, 2020). These reasons could explain why there was no effect of acids in gastric pH in our experiment. Nevertheless, their effect could be partially observed in the small intestine and cecum.

Our experiment showed a difference in the results obtained from the stomach tract, where no acid succeeded in lowering the pH, and the intestinal tract, where acids were more effective. The effectiveness of the use of acids on the intestinal tract has already been assessed in other studies, such as Cesari *et al.* (2008), where acids administration let higher fermentation activity in the intestinal tract compared to a basal diet without organic acid addition.

Data collected from previous works showed inconsistent results about gastric pH modulation: while some studies reported a significant reduction, others found no effects on gastric pH following dietary acidification. In a meta-analysis made on piglets where data from 22 studies using individual organic acids at high doses (1% to 2%) were collected, stomach pH was lower in 55% of the cases, higher in 36% and equal in 9% of the cases for acidified diets compared to control (Tung and Pettigrew, 2006). These inconsistencies among different studies may be because gastric contents are heterogeneous and there are different pH values in different stomach regions (related with methodological and measurement conditions and differences). In addition, the time of sampling can influence pH values as gastric pH is subjected to fluctuations over time after feeding (Clemens *et al.*, 1975).

It has already been assessed from previous works (e.g., Partanen and Mroz, 1999) that the interaction of treatments themselves, candidates, dietary and environmental factors give considerable variations in the results to organic acids supllementation, both on performance and physiological traits. However, the effects of organic acids on animals' performance often lack consistency and reproducibility (Dibner and Buttin, 2002). Thus, studies failing to report benefits of organic acids in livestock farming are also numerous (Falcão-e-Cunha *et al.*, 2007).

Nevertheless, our objective was to compare, as a screening test, the effects of different organic acids individually and at different doses. Following this approach, we can reject candidates (acids) and doses (pH) that do not benefit rabbit growth post-weaning and select the most adequate candidates that would be further tested in another long-term performance experiment. Combinations of them would potentially be conducted once the independent effects are precisely assessed in the screening

and performance phase. In any case, further studies need to be carried out to understand better acids' behaviour in young rabbits.

## 5. CONCLUSIONS

In the present study the administration of organic acids via drinking water did not improve growth performances of weaned rabbits in a significative way, but influenced their gastrointestinal pH values. Treatments with pH 3 tended to lower down body weight, average daily gain and average daily feed and water intake, while treatments at pH 4 and 5 did not show any detrimental effects. The results showed that treatments at pH 3, in particular ACET3, BUT3 and PROP3, did not meet the organoleptic preferences of rabbits and so their water ingestion was significantly lower than other treatments and consequently affected negatively their growth performance.

Acids administration has registered to be more effective in the intestinal tract rather than in the stomach, where buffering capacity of feed most influenced the effectiveness of acids. While almost all the acids have been helpful in the same way to reduce the intestinal pH values, in the gastric trait administering water seemed to be still the best way to maintain the pH level low.

Extending the supplementation duration of acids until fattening phase could lead to different results and be the key to enhance weight performance and health status of rabbits. However, the limited physiological development of the gastrointestinal tract of rabbits at weaning and how this condition could impact digestive efficiency have to be considered.

In conclusion, what could be taken out from this first experiment is that administering organic acids in rabbit farming is a strategical way to help a healthy gastrointestinal development at weaning phase; and could have some potential in enhancing performance parameters if acids supplementation is prolonged through time. Results obtained from current work could suggest the most adequate candidate organic acids to be implemented in growing rabbits were formic acid, acetic acid and citric acid. Candidate pH ranged from 4 to 5.

However, further studies on behaviour of acids' ingestion and effectiveness at weaning period in rabbit farming are necessary to define more precisely the relationship between acid intake and GIT health status and development. The amount of research on this topic is still limited in rabbits compared to other livestock species. Although results have often been inconsistent, several studies suggest that it is possible to develop a more defined and effective action plan in the use of organic acids in rabbit's nutrition.

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## 7. ANNEXES

### *7.1 FEED ANALYSES*

#### *7.1.1 Determination of DRY MATTER*

BACKGROUND:

Humidity is known as the proportion of free and combined water contained in food; the remaining part up to 100 is the dry matter.

Dry matter is determined by dehydration at 105°C up to constant weight. However, in these conditions some volatile substances also evaporate from the dry matter (default error) and others can oxidize and gain weight (excess error).

In case of food excessively high in volatile or alterable by heat materials other methods of analysis should be used.

MATERIALS:

- Drying stove
- Porcelain crucibles
- Metal tweezers
- Calcium chloride or similar
- Precision balance 0.0001 g

PROCEDURE:

The analysis is carried out in triplicate.

Dehydrate the porcelain crucibles (check that the crucibles are numbered, otherwise number them with a pencil) in a stove at 105°C for 10 to 12 hours.

Let them cool in a desiccator and weigh the empty crucibles (A).

Put in each crucible 3 g. of sample and weigh the crucibles with the sample (B), then store in the stove and dehydrate for at least 24 hours.

Remove the crucibles from the stove, let them cool in the desiccator and weigh again (C).

CALCULATION:

Known the weight loss corresponding to the amount of sample used, by a simple rule of three the percentage of dry matter is expressed:

$$Wet\ sample\ weight: D = B - A$$
$$Dry\ sample\ weight: E = C - A$$
$$\%MS = (E/D)\ x\ 100$$

#### *7.1.2 Determination of ASH and ORGANIC MATTER*

BACKGROUND:

Ash is the residue that remains after calcining the feed sample at 500°C to a constant weight, and is basically made up of oxides, carbonates, phosphates and mineral sulfates. At this temperature some inorganic components can evaporate. It is important that the temperature does not exceed 550°C because there would be large losses of chlorides.

Some samples may have a difficult combustion, in which case the burned product can be moistened with a few drops of hydrogen peroxide before calcining.

When the sample is very rich in sodium chloride (molasses, fishmeal) the metal compounds that make up the ashes must be transformed into sulfates. The abundance of sodium chloride manifested by decrepitations in the mass during calcination is a result of several non-matching determinations. In this case a previous combustion is carried out and the resulting carbonaceous product is moistened with a few drops of concentrated sulfuric acid and then calcined following the general procedure. The weight of the sulfated ashes must be multiplied by 0.9 to transform them into non-sulfated ashes.

The difference between dry matter and ash constitutes organic matter.

MATERIALS:

- Heating plate in extractor hood

- Incineration muffle
- Desiccation stove
- Porcelain crucibles
- Metal tweezers
- Desiccator of calcium chloride or similar
- Precision scale 0.0001 g

PROCEDURE:
The analysis is carried out in triplicate.
Number with a pencil and calcine the crucibles of porcelain, then store the crucibles in the dehydration stove, remove, let them cool in desiccant and weigh each empty crucible (A).
Put in each crucible 3 grams of sample and weigh (B).
Carry out a pre-combustion on the heating plate until ceases smoke's emission.
Put the crucibles into the muffle and calcine at 500°C for at least 3 hours, until you get white ashes.
Remove the crucibles from the muffle and put on the dehydration stove to cool a little, remove, let cool in desiccator and weigh again (C).

CALCULATION:
The calculation is made referring to dry matter of the sample.
Once is known the weight loss of the sample quantity used, the ash content is calculated by a simple rule of three:

$$Wet\ sample\ weight: D = B - A$$
$$Ash\ weight: E = C - A$$
$$MS = dry\ matter\ of\ the\ sample\ in\ \%$$
$$\%\ Cenizas\ (\%MS) = \{E/[(D\ x\ MS)/100]\}\ x\ 100$$
$$Organic\ Matter\ (\%MS) = 100 - \%Ash\ (\%MS)$$

*7.1.3 Determination of CRUDE FAT in rabbit meat*
BACKGROUND:
The method is based on the solubility of lipids in organic solvents (ethanol, ether, ecc.). For the total quantification of lipids in meat it is necessary to perform hydrolysis prior to fat extraction.

MATERIALS:
- Hydrolysis unit
- Organic solvent dispenser
- Soxtec extractor system
- Capsules and thimbles (3 boxes of 6 capsules each)
- Receptor vessels
- Degreased cotton
- Soxcap Filters
- Plastic rubbers
- Gloves

REAGENTS:
- HCI 6N: for each analysis approximately 1l is required. If missing, perform a 1:2 dilution with HCl 12N. Measure 500 ml of HCI 36-38% (12N) and add 500 ml of distilled water.
- Celite 545
- Diethyl ether

PROCEDURE:
*Acid hydrolysis (Soxcap 2047) FosHotplate 2022 Equipment:*

Hydrolysis is performed to separate the phospholipids from the sample, and thus quantify them together with the triglycerides. If the sample had many triglycerides and the phospholipid part is negligible, this step would not be necessary.

1. Place a filter in each capsule.
2. First weigh the capsule with the filter on the precision balance. Then weigh 1.5 g of lyophilized sample (3 g if the sample is wet, previously grounded). Write down the exact weight, from here the fat is get.
3. Weigh approximately 0.35 g of Celite so that the sample does not clog. This weight does not need to be written down.
4. In the hydrolysis equipment, add HCI 6N (fill up to a little more above the "R" line).
5. Introduce the carousel with the samples into the hydrolysis container. The acid should cover the sample well, and if not, add HCL with a glass funnel until covering it.
6. Cover the container with plastic rubbers. Place the suction tube in the central straw of the carousel and turn on the water tap to cool down with cold water.
7. Turn on the heating plate at maximum power and leave the container for about 15 minutes until starts to boil. Then, lower the power of the plate and leave the container for 60 minutes. After 60 min turn off the heat.
8. Open the waste removal valve to remove the residue (black liquid). Wait until the container is emptied.
9. Once empty, open the rinse water valve and wait until the water fills the container to the level of the carousel.
10. Perform 5 washes.
11. After the 5 washes and with the empty container, close the water tap, remove the carousel and let the capsules drain on LAB paper (switching to a new paper 3-4 times).
12. Place a special degreased cotton on top of the capsule.
13. Take the receptor metal cups and leave the receptor vessels and the capsules in a clean carousel and store in the stove at 90°C overnight.

*Extraction (Soxtec 2055):*

1. Remove the receptor vessels and capsules from the stove. Put the metal vessels in the desiccator and let them cool for about 15 minutes.
2. While the glasses are cooling down, turn on the cooling and heating of the Soxtec equipment.
3. Prepare the capsules for Soxtec:
    - Push the filter on one side to introduce it inclined in the center of the capsule
    - Screw a cartridge on the end that fits
    - Put a metal magnet ring in each capsule
    - Place degreased cotton at the base of the capsule
4. After 15 min, weigh the glasses (taking the desiccator next to the precision scale) and write down the exact weight on the template. The fat is collected in these glasses, so by difference with this weight the amount of fat is taken out
5. Assemble the Soxtec:

Left lever: move capsules
Right lever: move glasses

- The capsules are placed in the Soxtec thanks to the metal ring and magnet of the Soxtec. At the end, raise the capsules (left lever) to the top so that the glasses can fit.
- Place the tray of the receptor vessels in the Soxtec. Lower the left lever to the intermediate position and lower the right lever to the end. Close the window.
- Fill the receptor vessels with 90 ml of dimethyl ether and count 25 minutes of recovery.
- After 25 minutes, take the tray out of the cups and leave the cups in the stove at 100°C for 2 hours. Take the capsules and cartridges out so that the ether evaporates for a while (30min-1h). Then, throw the filter and cottons out of the capsules and wash them.

6. Extraction:

- Procedure: Mix ether and sample, wash and recollect the ether.

- Lower the two levers to the maximum (the one of the glasses must already be lowered). Press the ON button and wait a few seconds for the temperature to arrive. Hit the alarm button and the 30 minutes boiling phase begins.
- After 30 minutes place the lever on the left in the intermediate position and count 60 minutes (rinsing phase).

7. Weigh the glasses with the fat.

CALCULATION:

$$\%lipids = \frac{(glass\ weight + fat) - vessel\ weight}{sample\ weight} x100$$

*7.1.4 Determination of CRUDE PROTEIN (Dumas)*

BACKGROUND:

In recent years, the Kjeldahl method has been progressively replaced by the Dumas method, because it is faster and does not use hazardous chemical reagents.

On the other hand, the results obtained by the Dumas method are slightly higher than those of the Kjeldahl method. This is because the Dumas method measures almost all the nitrogen that is not part of the proteins, while the Kjeldahl method only measures a part. (UNE-EN 16634.1.2009)

Considering that the protein content of a product calculated by both methods is only an approximation to the actual value, the choice of which to use between the two is left to personal discretion. The most appropriate solution is to use a second factor that eliminates the systematic errors caused by the nitrogen content that is not part of the protein for each different product. However, this second factor must be determined for each product, just like the existing factors that show the ratio of protein to the total nitrogen content. (UNE-EN 16634.1.2009)

The elemental analyzers by combustion (Dumas method) analyze the entire element in the sample, in this case the nitrogen. The sample is burned at high temperature so that all the organic molecules are thermally broken, releasing the elements they contain such as C, H, N, S and O. These ones are oxidized, thanks to the input of excess oxygen, immediately to CO2, SO2, NOx and H2O, in order to be detected later in specific cells (called detectors) for each element to be measured. In the specific case of N2, the cell is a thermal conductivity detector (TCD).

The Dumas method has a great versatility and is indicated for the analysis of natural organic products, animal feed and derivatives in general, among which stand out: raw materials for feed, milk, juices, serums, leachates, manure and urine.

MATERIALS:

- Elemental analyzer LECO CN628
- Spatula
- Precision scale 0.0000 g
- Tin papers (for solid samples)
- Tin capsules (for liquid samples)

REAGENTS:

- EDTA (Ethylene diamine tetracetic acid)
- Lecosorb (sodium hydroxide in non-fibrous silicate)
- Anhydrone (magnesium perchlorate)
- Quartz wool
- Glass wool
- Metal wool
- Metallic copper
- Porous crucible

PROCEDURE:
Open the gases O2, air and He (helium) if closed (in this case some previous time is needed to stabilize the TCD cell). Do a system check to assess everything is OK.
Proceed with a leak test if a change in the reagents, crucible or anything related to the maintenance of the equipment has been carried out before, as if the equipment has not been analyzed for a long time. Check for a stable resting and working temperature as well.
It is also advisable to burn 1 or 2 samples of EDTA for eliminate remains that could remain in the oven of the previous working day and discard them, since they are only for preconditioning the analyzer.
"Campione bianco" si riferisce all'utilizzo del campione per azzerare uno strumento durante una procedura di analisi. Un bianco campione può correggere possibili errori dovuti al colore o alla torbidità di un campione in un dato momento prima di aggiungere i reagenti
Blank samples: First it's necessary to assess the best working method for the analysis of the matter that wanted to be carried out from the problem samples. Once the method is selected, the necessary blank samples are identified to maintain the equipment stable according to the expected level of nitrogen of problem samples. When the area of N reaches the expected value, it is possible to start the activity, since the expected value can oscillate based on the number of blank samples. The %N measured in each application may be different due to the calibration curves and their ordinate at the origin, since is not possible to lower the %N measured in blank samples below the ordinate at the origin of its calibration.
As a general reference of the nitrogen blank sample, is normal for it to stabilize at a value below 5 areas and it is stable when the variation of 3 consecutive blank samples is not greater than 0.4 areas between them.
An area value of 4 represents approximately 0.01% N concentration in a 100 mg sample. This is corrected with the correction of the blank sample.
A variation in the blank sample's areas of 0.15 supposes approximately a variation of 0.015% of N concentration in a sample of 100 mg and this could not be corrected with the correction of the blank sample.
Whenever one working method is changed with another one on the same day, at least one blank sample must run through the new method.
Once the blank sample is stable, it is incorporated into the system so that it applies the correction of the blank samples in the calculations.
Calibration drift's establishment: once the equipment is stabilized at the value of the desired blank sample for the application, the reference model must be analyzed as drift (EDTA or barley). Thus, 2 to 3 samples of drift model are analyzed from the selected method and, looking at the results obtained, it is decided whether it's necessary to correct the calibration curve with the new drift or not, then it's possible to perform the analysis of problem samples.
Weigh samples: the samples are weighed depending on the expected protein. For most raw materials and feed are weighed 0.2 g, and for concentrated samples > 40% PB are weighed 0.18 g. For liquid samples the ideal volume is 0.5 ml. Solid samples are weighed using tin papers, liquids samples using tin capsules.
Analysis: On average, the duration of the analysis is about 5 minutes per sample. The analysis is usually performed in duplicate, and it is taken for granted if the RSD% is greater than 0.05%. Once obtained the required value of RSD%, a replica is run to establish the average score.
Calibration: it is done by analyzing increasing amounts of a known %N, EDTA or barley standard, depending on the samples' content of nitrogen (high or low). Thus, there are two calibration curves or lines (type $y = a + bx$) and two working methods associated with those lines (high and low nitrogen).

CALCULATION:
The equipment directly gives as outputs the %N from the sample or the % of crude protein if the correction factor of 6.25 or 6.38 (for milk) is introduced.
The calculations are performed by applying the equation of the calibration line like $y = a\ bx$.

*7.1.5 Determination of VAN SOEST FIBERS (ANKOM System)*
BACKGROUND:
The different fractions determined using the Van Soest method are estimators of the different structural carbohydrates and indigestible substances linked to them that form vegetable cell wall.

The sequenced analysis separates three fractions according to their digestive utilization. The first is the Neutral Fiber-Detergent (FND) fraction, that is the proportion of insoluble food in a solution of lauryl-sodium sulfate detergent at pH 7 after gentle boiling for 1 hour. FND represents the vegetable cell wall without pectins, which are completely solubilized in such a solution and so are easily digestible compounds.
This non-soluble cell wall is constituted by two fractions: one less lignified and more digestible that includes the hemicelluloses, and another more lignified and indigestible that is called Acid-Detergent Fiber (FAD), which is the proportion of the insoluble food in a detergent solution in medium acid after 1 hour boiling and corresponds to the lignocellulose. FAD also includes cutin, which also is indigestible for animals and is found in small proportions, some compounds lignified with nitrogen and some altered proteins. The difference between FND and FAD, both ash-free, gives the estimate content of the hemicellulose.
The last fraction separated is the detergent-lignin acid (ADL) which is the proportion of the ash-free residue insoluble in sulfuric acid at 72% after 3 hours of contact. This treatment solubilizes cellulose and therefore corresponds to lignin (cutin is also included).
Since the publication of the original techniques in 1963, there have been some modifications to the method. The most important is the prior determinations with alpha-amylase to avoid the retention of starch during the analysis and to avoid the retention of proteins of the cellular content in the FND.

MATERIALS:

- Digestion apparatus-ANKOM^200/220 FIBER ANALYZER
- ANKOM F57 filtration bags
- Filtration system-ANKOM TECHNOLOGY-FILTRATION BAGS F57
- Heat sealer-ANKOM TECHNOLOGY-1915/1920
- Desiccator-ANKOM TECHNOLOGY- (Anti-humidity bag to store the filtration bags before weighing them-F39)
- Calcium chloride desiccator or similar
- Spatula
- Precision scale of 0.0001 g
- Drying stove
- Oven or Muffle
- Porcelain crucibles (without enameled base)
- Beakers of 500, 2000 and 3000 ml

REAGENTS:

- Thermoset alpha-amylase solution (Ref: A-3306 Sigma). Store refrigerated at +4°C
- Ethylene diamine tetra acetic (disodium salt, PM=372.24). (ATTENTION: dangerous product)
- Sodium tetraborate 10-hydrate (PM=381.37)
- Sodium lauryl sulfate (dodecylhydrogensulfate sodium salt, PM=288.38) (ATTENTION: dangerous product)
- Anhydrous disodium phosphate (PM=141.96)
- Triethylene glycol (PM=150.18). (ATTENTION: dangerous product)
- Cetyltrimethylammonium bromide (PM=364.45). (ATTENTION: dangerous product)
- SO4H2 concentrate (d=1.84). (ATTENTION: highly corrosive product)
- Anhydrous sodium sulfide (Na2SO3)

SOLUTIONS:

- <u>FND Solution:</u> Weigh 37.22 g of ethylene diamino tetra acetic (disodium salt) and 13.62 g of sodium tetraborate. Transfer to a 2000 ml volumetric flask and dissolve with distilled water under slight heating. Weigh 60 g of sodium lauryl sulfate and 9.12 g of anhydrous disodium phosphate. Pour them into the flask and dissolve. Add 20 ml of triethylene glycol and rinse with distilled water. Check that the pH is between 6.9 and 7.1.

- FAD Solution: Weigh 40 g of cetyltrimethylammonium bromide (PM=364.45). Transfer to a 2000 ml volumetric flask, dissolve and rinse with SO4H2 1N (add 55 ml of concentrated H2SO4 to a 2000 ml volumetric flask and rinse with distilled water).

- 72% SO4H2: Dissolve 700 ml of SO4H2 in 300 ml of distilled water. Check that d=1,634. (ATTENTION: it gets very hot when mixed, cool down in a cold-water bath).

PROCEDURE:
The analysis is performed in duplicate. A blank sample (empty bag) that receives the same treatment as the sample is used.
*FND Determination:*
Weigh the filter bag (ANKOM F57) and calibrate the scale. The bags have a negligible moisture content and do not need to be dried previously, unless they are stored outside without the desiccant in a high humidity environment.
Weigh 0.5 g (+/- 0.05 g) of ground sample, directly into the filtration bag.
Seal the bag 0.5 cm from the open end using the heat sealer (ANKOM 1915 or 1920).
Spread the sample evenly inside the bag by shaking it, to avoid caking.
In samples with a content of soy products or >5% fat content, it is necessary to extract the fat from the samples by placing 24 bags filled with samples in a 500 ml glass with lid and add enough acetone to keep the bags submerged, then secure the lid. Shake the glass 10 times and leave the bags submerged for 10 min. Repeat with new fresh acetone. Pour the acetone into a waste container and let the samples sit for 5 minutes at dry air on a metal sieve.
*Exception:* roasted soybeans. Due to the special properties of Roasted Soy it is necessary to perform a fat extraction for a longer time. To do this, place the bags of Toasted Soybeans in a 500 ml glass with a lid. Add enough acetone to cover the bags and close the lid. Shake the glass 10 times and drain out the acetone. Add new fresh acetone and leave the samples submerged for 12 hours. After this time, drain out the acetone and let the bags dry to air.
Place the 24 bags in the bag suspensor (ANKOM F11). Place three bags per basket (8 baskets in total). Place the baskets on the shaft rotated 120 degrees each of them. The 9th basket is empty.
Introduce the bag suspensor in the digestion tank of the ANKOM Analyzer. Place the weight on top of the 9th basket to keep the bag suspensor submerged. Close the drain valve of the digestion tank.
Add 1900-2000 ml of FND solution at room temperature into the digestion tank. If less than 20 bags are used add 100 ml per bag of FND solution. Add 20 g (0.5 g/50 ml of FND solution) of sodium sulphite to the solution in the reaction tank and 1 ml of thermoset alpha-amylase enzyme. Program the clock for 75 minutes, turn on stirring and heating. Check that the bag suspensor is shaking and close the digestion tank tightly. Start the programmer.
After 75 minutes (alarm rings) turn off the stirring and heating. Open the drain valve and let the hot solution out before opening the lid.
After evacuating the solution, close the valve and open the lid. Add approximately 1900-2000 ml of hot water (90-100°C) for washing out, turn on the agitator and leave the heating turned off. Close the lid but not firmly. Shake the bags in the washing water for 5 minutes. Repeat the wash 2 more times (3 times in total).
Remove the filter bags from the suspensor and gently remove water excess. Place the bags in a 250 ml glass and add acetone to cover the bags. Leave the bags submerged for about 3 minutes, remove them and carefully remove acetone excess.
Place the bags outside and let dry out to air. Make sure the bags are well air-dried before putting them into the desiccation stove.
Complete drying in a drying stove at 103°C for 4 hours.
Remove the bags from the stove and place them in a desiccator (ANKOM or calcium chloride type), until they reach room temperature.
Weigh the bags.

*FAD Determination:*
Place the bags in the suspensor and put it in the reaction tank, as done in the FND determination.

Add 1900-2000 ml of the FAD solution into the digestion tank. Program the clock for 75 minutes, turn on the stirring and heating. Check that the bag suspensor is shaking and close the digestion tank tightly. Start the programmer.
After 75 minutes (alarm rings) turn off the stirring and heating. Open the drain valve and let the hot solution out before opening the lid.
Do the washing as it was done for the FND solution.
Remove the filter bags from the suspensor and gently remove water excess. Place the bags in a 250 ml glass and add acetone to cover the bags. Leave the bags submerged for about 3 minutes, remove them and carefully remove acetone excess.
Put the bags outside and let them dry out to air. Make sure the bags are well air-dried before putting them in the desiccation stove.
Complete drying in a drying stove at 103°C for 4 hours.
Remove the bags from the stove and place them in a desiccator (ANKON or calcium chloride t), until they reach room temperature.
Weigh the bags.

*ADL Determination:*
Place the dry bags with the samples in a large 3I tank and add enough quantity (approximately 250 ml) of 72% sulfuric acid to cover the bags.
*IMPORTANT:* The bags must be completely dry and at room temperature before adding the concentrated acid. If there is humidity in the bags, the heat generated by the reaction between H2SO4 and H2O would affect the results (the sample inside the bag would be charred).
Place a 2l glass inside the 3l tank to keep the bags submerged. Shake the bags at the beginning and every 30-minutes intervals by pushing and lifting the 2l glass up and down for approximately 30 times.
After 3 hours drain out the sulfuric acid in a waste container and wash with hot water (90-100 °C) to remove all the acid excess. Repeat the washes until the pH is neutral. Wash with approximately 250 ml of acetone for 3 minutes to remove the water. Do not place the bags on the stove until the acetone evaporates completely.
Dry completely the bags in a drying stove at 103°C for 4 hours.
Remove the bags from the stove and place them in a desiccator (ANKON or calcium chloride type), until they reach room temperature.
Weigh the bags.
Incinerate the bags filled with sample in porcelain crucibles (desiccated and weighed previously) for 5 hours in muffle at 550°C.
Keep the crucibles in the stove at 103°C for 1 hour. Cool in a desiccator and weigh the crucibles for the calculation of the ashes.

CALCULATIONS:
A = weight of the blank sample bag
B = weight of the empty sample bag
D = weight of the sample
Pfnd = weight of the bag with the sample after extraction with the FND solution and desiccation
Pfad = weight of the bag with the sample after extraction with the FAD solution and desiccation
Padl = weight of the bag with the sample after extraction with the ADL solution and desiccation
MS = dry matter of the sample in %
Cb= ashes of blank sample= *[(Crucible + blank sample's ashes) - crucible weight]*
Cm = ashes of the sample = *[(Crucible + sample's ashes) - crucible weight]*

$$\%FND\ (\%MS) = \frac{(Pfnd - Cm) - [B\ x\ (1 - Cb/A)]}{(D\ x\ MS)/100}\ x100$$

$$\%FAD\ (\%MS) = \frac{(Pfad - Cm) - [B\ x\ (1 - Cb/A)]}{(D\ x\ MS)/100}\ x100$$

$$\%ADL\ (\%MS) = \frac{(Padl - Cm) - [B\ x\ (1 - Cb/A)]}{(D\ x\ MS)/100}\ x100$$

$$\%Hemicelluloses\ (\%MS) = \%FND - \%FAD$$
$$\%Celluloses\ (\%MS) = \%FAD - \%ADL$$

## *7.2 TREATMENTS PREPARATION:*

**Materials:**

- Distilled water
- Plastic tubes 2000mL
- Plastic tubes 100mL
- Plastic beaker 2000mL
- Plastic funnel
- Thermometer
- Pipette Pasteur
- Graduated pipette 250µl
- pH meter

### *7.2.1 PRE-WORK: Dissolution of Citric acid (CIT)*

Pure Citric acid (CIT) comes in the form of solid powder 99%; reference C0759 Sigma-Aldrich. pH=1.7 (100g/L); pKa=3.13.
**Objective:** Determine the required time and the water temperature for the dissolution of citric acid.
**Performance:** Dissolve 100g of citric acid in 1000mL of tap water (pH≈7.5). Heat the solution on a hotplate with stirring. The pH of the mixture should be around 2. Verify if the pH remains stable in the following days, if not, it is necessary to dissolve the acid each day in which is changed the water from the drinkers.

### *7.2.2 Treatments' Dosage test*

**Objective:** Determine the pH curve with the addition of increasing doses of organic acids in water. Three different pH are reached for each treatment: pH 3, pH 4, pH 5.
**Performance:** Fill a beaker with 500mL of tap water at 25°C (pH≈7.5) and add 125µl of acid (0.125% of acid) in a variable range of additions with a graduated pipette of 250µl under continuous stirring. With a pH meter measure the pH to assess the pH curve of each acid at each dosage. From *Figure 7.1* it is possible to see that the pH curve of each treatment shows an exponential descending development with an initial sharp fall with the addition of just a few mL of acid. This decline tends to flatten out as the pH continues to lower down as a response to the continuous addition of acid.

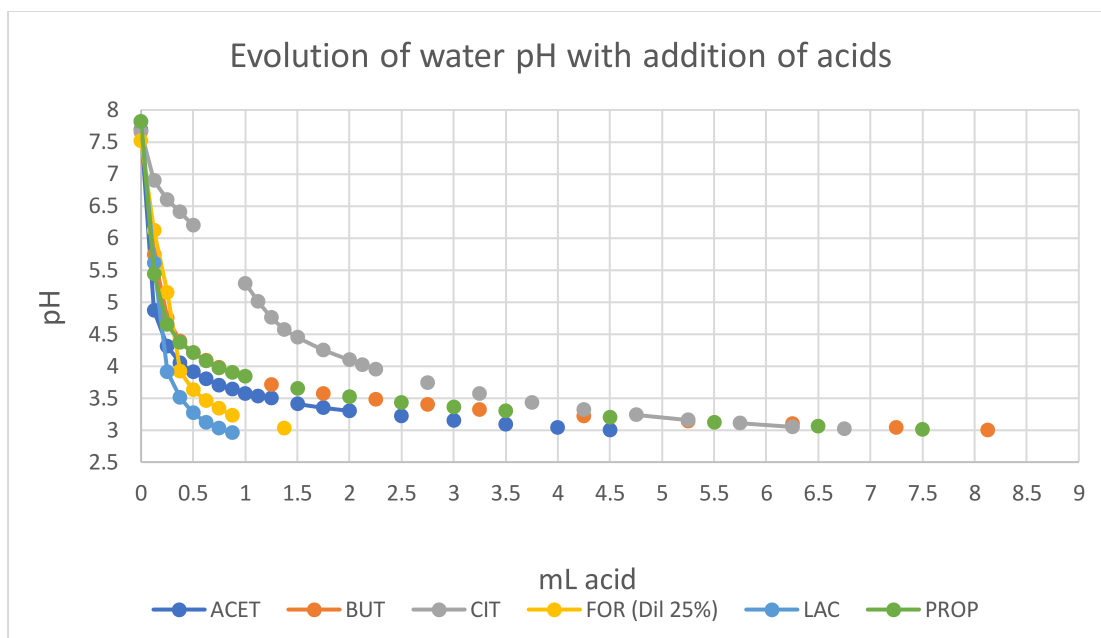


*Figure 7.1* – water pH curves' evolution with increasing doses of acids.

The final dosages of acids are shown in *Table 7.1* and *Table 7.2*. In Table are shown the final dosages obtained in the laboratory. In Table are shown the dosages used in the farm to fill the bottle-drinkers.

*Table 7.1* – Acids' final dosage in 500 mL of tap water.

| Final dosage | pH tap water=7.65 | | |
|---|---|---|---|
| mL of acid in 500 mL tap water | pH 5 | pH 4 | pH 3 |
| ACET | 0.125 | 0.375 | 4.500 |
| FOR (Dil. 25%) | 0.250 | 0.375 | 1.375 |
| CIT | 1.125 | 2.125 | 6.750 |
| BUT | 0.200 | 0.750 | 8.125 |
| PROP | 0.200 | 0.750 | 7.500 |
| LAC | 0.160 | 0.250 | 0.875 |

*Table 7.2* – Acids final dosage in 2000 mL of tap water. These are the doses used to fill the drinkers in the farm during the experimentation.

| Final dosage | pH tap water=7.65 | | |
|---|---|---|---|
| mL of acid in 2000 mL tap water | pH 5 | pH 4 | pH 3 |
| ACET | 0.50 | 1.50 | 18.00 |
| FOR (Dil. 25%) | 1.00 | 1.50 | 5.50 |
| CIT | 4.50 | 8.50 | 27.00 |
| BUT | 0.80 | 3.00 | 32.50 |
| PROP | 0.80 | 3.00 | 30.00 |
| LAC | 0.64 | 1.00 | 3.50 |

*7.2.3 Treatments' Stability test*

**Objective:** Determine the stability of pH3, pH4, pH5 through time.

**Performance:** Basing on the activity number 2, pour in a plastic glass of 125mL a sample of each treatment at each pH (19 samples in total). Measure and record the pH at zero time and at 6, 24, 48 and 72 hours later with a pH meter. Water should always be at the temperature of 25°C.

The records of the stability test are shown in *Table 7.3*.

*Table 7.3* – Treatments' stability test records.

| **Date** | **Hour** | | **Hour** | | | |
|---|---|---|---|---|---|---|
| **28/03/2022** | **14:00** | | **20:00** | | | |
| **ACID** | **pH 3** | | **pH 3** | | | |
| **ACET** | 2.98 | | 3.04 | | | |
| **BUT** | 3.01 | | 3.06 | | | |
| **CIT** | 3.03 | | 3.06 | | | |
| **FOR** | 2.95 | | 3.02 | | | |
| **LAC** | 2.95 | | 3.01 | | | |
| **PROP** | 3.01 | | 3.05 | | | |
| **CON** | | | | | | |
| **29/03/2022** | **10:00** | | **14:00** | | | |
| **ACID** | **pH 3** | | **pH 3** | **pH 4** | **pH 5** | |
| **ACET** | 3.02 | | | 4 | 5.04 | |
| **BUT** | 3.06 | | | 4.03 | 5.13 | |
| **CIT** | 3.06 | | | 4.04 | 4.91 | |
| **FOR** | 3.02 | | 3.03 | 3.86 | 4.95 | |
| **LAC** | 3.04 | | | 3.9 | 4.92 | |
| **PROP** | 3.04 | | | 4 | 4.7 | |
| **CON** | | | | | | 7.5 |
| **30/03/2022** | **15:00** | | | | | |
| **ACID** | **pH 3** | **pH 4** | **pH 5** | | | |
| **ACET** | 3.04 | 3.96 | 5.01 | | | |
| **BUT** | 3.04 | 4.03 | 5.22 | | | |
| **CIT** | 3.03 | 3.97 | 4.94 | | | |
| **FOR** | 3.04 | 3.9 | 5.15 | | | |
| **LAC** | 2.99 | 3.9 | 5.03 | | | |
| **PROP** | 3.04 | 4 | 4.65 | | | |
| **CON** | | | | 7.73 | | |
| **31/03/2022** | **15:00** | | | | | |
| **ACID** | **pH 3** | **pH 4** | **pH 5** | | | |
| **ACET** | 3.03 | 3.98 | 5.06 | | | |
| **BUT** | 3.03 | 4.02 | 5.24 | | | |
| **CIT** | 3.02 | 3.98 | 4.98 | | | |
| **FOR** | 3.03 | 3.91 | 5.29 | | | |
| **LAC** | 2.99 | 3.88 | 5.15 | | | |
| **PROP** | 3.04 | 4 | 4.67 | | | |
| **CON** | | | | 7.89 | | |
| **01/04/2022** | **12:30** | | | | | |
| **ACID** | **pH 3** | **pH 4** | **pH 5** | | | |
| **ACET** | 3.02 | 3.93 | 5.02 | | | |
| **BUT** | 3.05 | 4.03 | 5.31 | | | |
| **CIT** | 3.08 | 4.01 | 5.05 | | | |
| **FOR** | 3.08 | 3.91 | 5.52 | | | |
| **LAC** | 2.99 | 3.88 | 5.24 | | | |
| **PROP** | 3.02 | 3.98 | 4.64 | | | |
| **CON** | | | | 7.94 | | |

In *Figure 7.2*, *7.3* and *7.4* the recorded data from the treatments' stability test are grouped by the pH. In *Figure 7.5* the curve's development of the Control treatment is shown. All the treatments tend to remain stable and close to their original pH, in particular treatments with pH 3 (the strongest ones). To avoid biases during the experiment due to acids' behaviour and to the increasing temperature of the external environment, treatments were prepared again every two/three days.

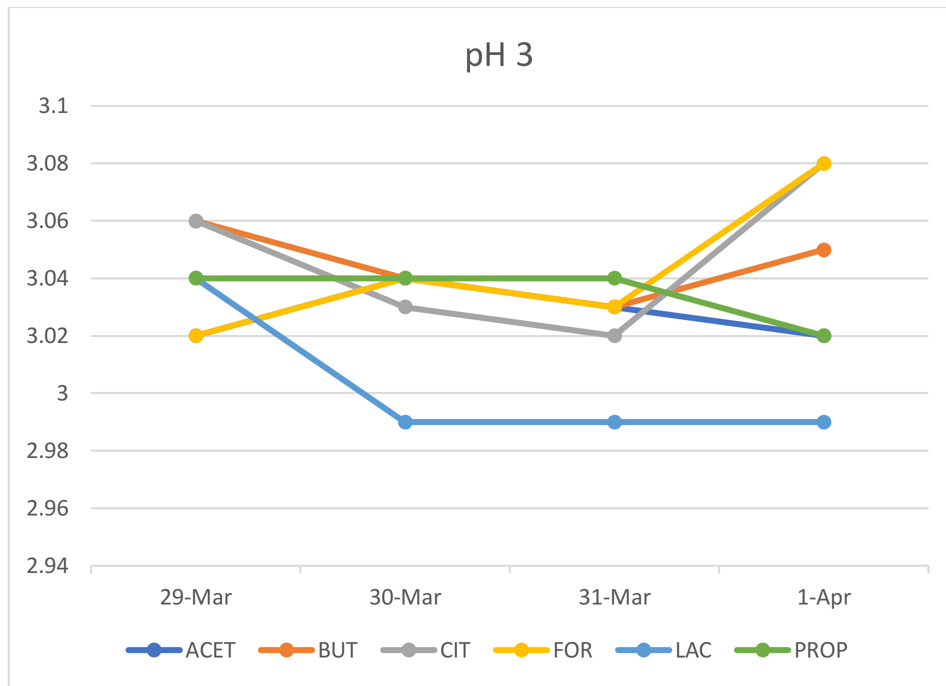


*Figure 7.2* – Treatments' stability test grouped by pH 3. The graph showed the stability of pH through days of treatments at an initial pH of 3 (i.e. ACET3, BUT3, CIT3, FOR3, LAC3, PROP3)

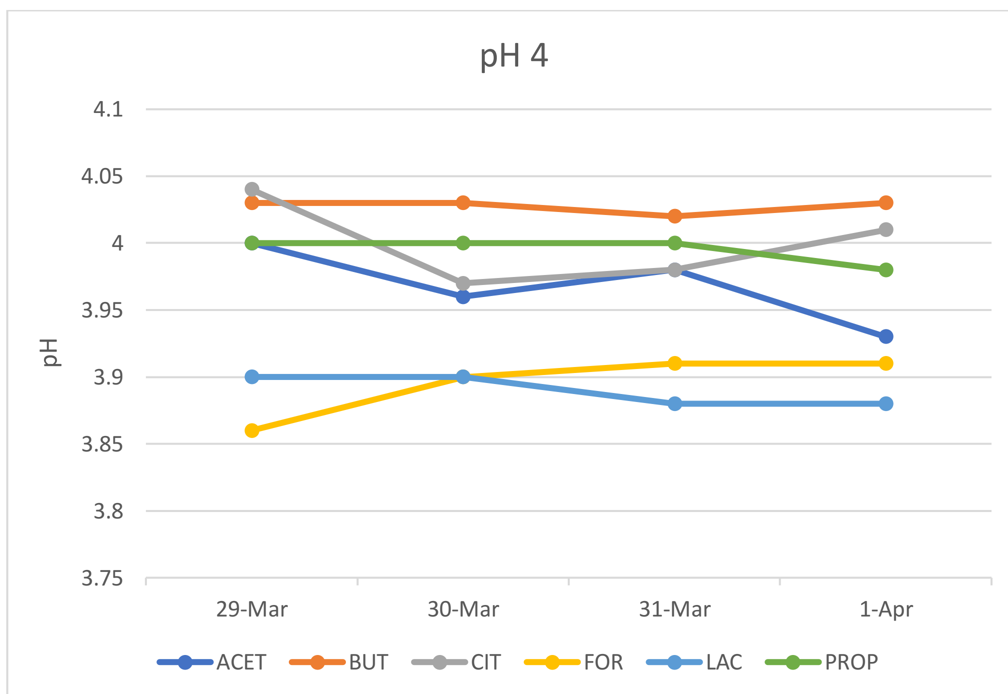


*Figure 7.3* – Treatments' stability test grouped by pH 4. The graph showed the stability of pH through days of treatments at an initial pH of 4 (i.e. ACET4, BUT4, CIT4, FOR4, LAC4, PROP4)

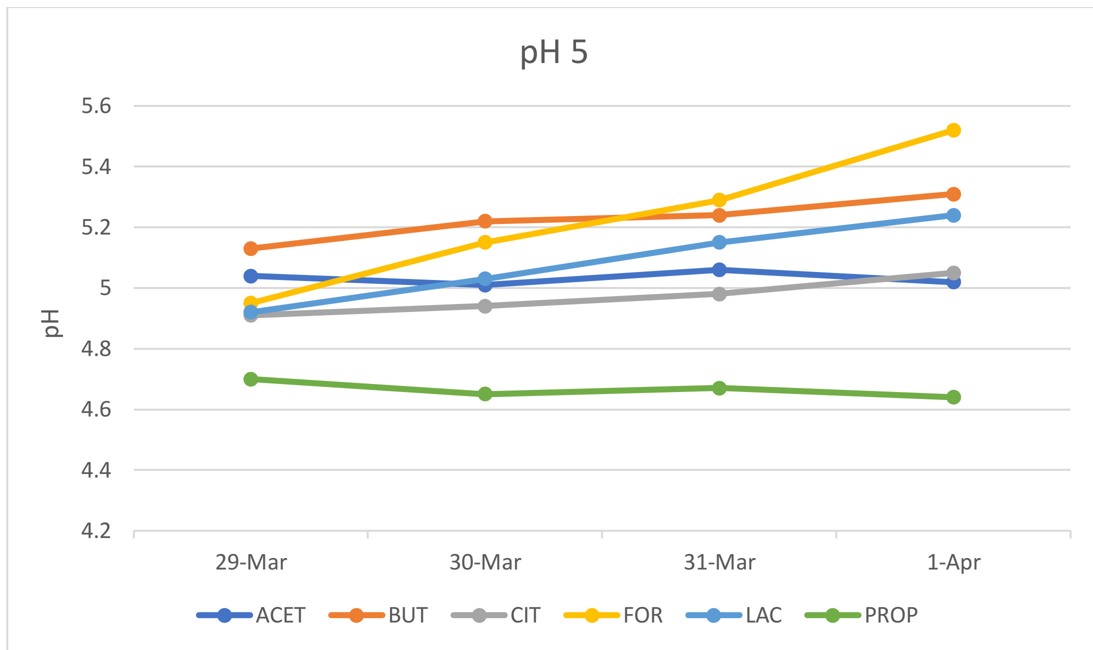


*Figure 7.4* – Treatments' stability test grouped by pH 5. The graph showed the stability of pH through days of treatments at an initial pH of 5 (i.e. ACET5, BUT5, CIT5, FOR5, LAC5, PROP5)

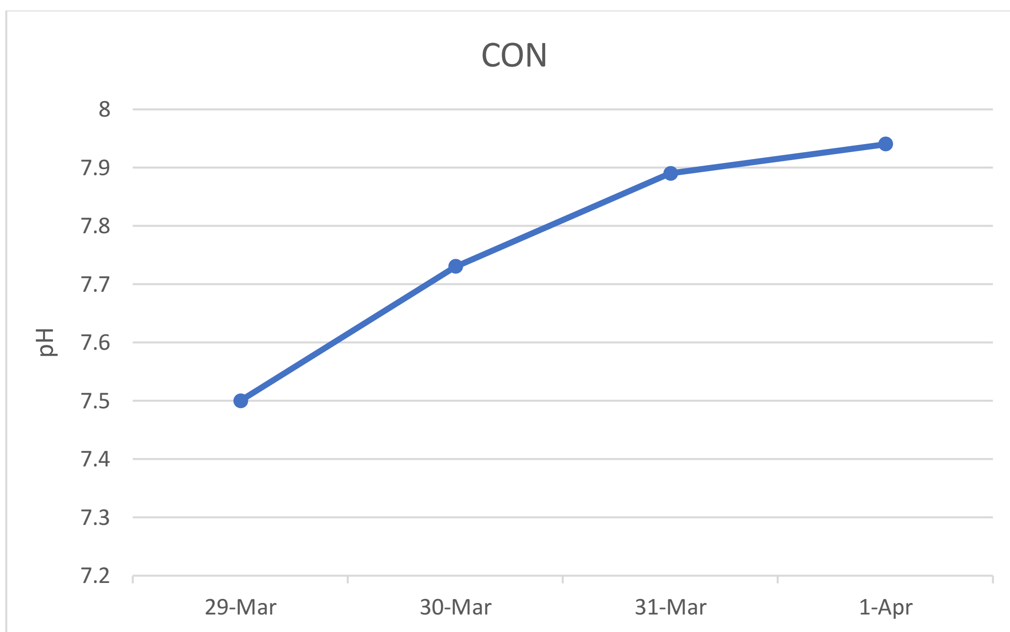


*Figure 7.5* – Water stability test. The graph showed the stability of water's pH through days.