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# EFFECT OF LEVEL OF DIETARY SOLUBLE FIBRE AND THREONINE ON DIGESTION AND GROWTH PERFORMANCE IN POST-WEANING RABBITS

**Thesis presented as a requirement for aspire to academic degree de Máster of Science en Nutrición animal:**

**Cecilia Alexandra CASTILLO MARTINEZ**

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## ABSTRACT

The aim of this work was to study the effect of soluble fibre and threonine deficiency on digestion and performance after weaning in rabbits. Four diets in a 2 x 2 factorial arrangement were used with two level of soluble fibre (89 vs.119 g/kg) and two level of threonine (5 vs. 6.4 g/kg). The effect of soluble fibre was studied substituting alfalfa hay (100 g/kg) by sugar beet pulp (126 g/kg), and the level of threonine was obtained supplemented L-threonine (0 vs. 1.42 g/kg). In the first experiment, 128 mixed-sex weanling rabbits of 35-d of age were used to determine the apparent faecal digestibility of DM and CP from 42 to 46 d (12/diet), the apparent ileal digestibility of DM and CP (at 46 d. 20/diet) and mucosa morphology (10/diet). In the second experiment, 140 weanling 25 d old rabbits of both sexes were used to determine the apparent faecal (from 32 to 35 d. 11/diet) and ileal (at 35 d. 20/diet) digestibility of DM, CP, TDF, starch and mucin concentration, mucosa morphology (10/diet) and growth traits (35/diet). In both experiments rabbits were fed with the experimental diets for 10 days and then they were slaughtered. In the first experiment rabbits fed with the high level of soluble fibre showed higher relative weight of total digestive tract and caecum and a lower caecal pH than those fed with a low level ($P \leq 0.032$), with no effect on growth traits. The increase of soluble fibre level in the diet led to a higher villous height/crypt depth ratio ($P < 0.001$) and increased the number of goblet cells per villi ($P<0.001$). Threonine level did not affect any trait. In the second experiment, mortality decreased with the high level of soluble fibre ($P = 0.002$), and tended to be reduced with threonine level ($P = 0.091$). The increase of soluble fibre level also increased the villous height/crypth depth ratio ($P < 0.001$), the number of goblet cells ($P = 0.008$) and the ileal and faecal flow of mucins ($P \leq 0.033$). However, no effect of threonine level on these traits was detected, but a positive effect on ileal starch digestibility in low soluble fibre diet was found ($P < 0.001$). The ileal and faecal digestibility of TDF increased with soluble fibre level ($P \leq 0.065$), with no effect on organ weights. In conclusion, the increase of soluble fibre improved intestinal mucosa integrity and mucin secretion leading to a better health status of the rabbits when the sanitary conditions worsened. Mucosa barrier traits were not affected by dietary threonine level although in poor sanitary conditions a low threonine level impaired rabbit health suggesting a limiting status of this amino acid.

## RESUMEN

El objetivo de este trabajo fue estudiar el efecto de un pienso deficitario en fibra soluble y treonina sobre la digestión y rendimiento en conejos post destete. Para ello se formularon cuatro piensos experimentales con un arreglo factorial 2 x 2 usando dos niveles fibra soluble (89 vs.119 g/kg MS) y dos niveles de treonina (5 vs. 6.4 g/kg MS). El efecto de la fibra soluble fue estudiado mediante la sustitución de heno de alfalfa (100 g/kg) por pulpa de remolacha (126 g/kg), y el nivel de treonina suplementando L-treonina (0 vs. 1.42g/kg). En el primer experimento, se utilizaron 128 gazapos destetados a los 35 días de edad, y se determinó la digestibilidad fecal aparente de la MS y proteína bruta (PB) desde los 42 a los 46 días (12/pienso) y la morfología de la mucosa (10/pienso). En el segundo experimento, se utilizaron 140 gazapos destetados a los 25 días de edad, y se determinó la digestibilidad fecal aparente (desde los 32 a 35 días de edad) e ileal (a los 35 días de edad) de la MS, PB, fibra dietética total y almidón. Además, se cuantificó la concentración de mucinas, la morfología de la mucosa (10/pienso) y los rendimientos productivos (35/pienso). Los piensos experimentales fueron suministrados durante 10 días, tras los cuales los animales fueron sacrificados. En el primer experimento, los animales que recibieron los piensos con un mayor nivel de fibra soluble mostraron un mayor peso relativo del tracto gastrointestinal y del ciego, y un menor pH cecal en comparación con aquellos animales alimentados con los piensos con un bajo nivel de fibra soluble ($P \leq 0.032$), sin observarse efecto alguno en el rendimiento productivo. Un mayor nivel de fibra soluble en la dieta condujo a un incremento del ratio longitud de villi/profundidad de cripta ($P<0.001$) y del número de células caliciformes por villi ($P<0.001$). Los niveles de treonina no afectaron a ninguno de estos parámetros. En el segundo experimento, un mayor nivel de fibra soluble redujo la mortalidad ($P = 0.002$) y un nivel adecuado de treonina también tendió a disminuirla ($P = 0.091$). Un mayor nivel de fibra soluble incrementó el ratio longitud de villi/profundidad de cripta ($P < 0.001$), el número de células caliciformes ($P = 0.008$) y el flujo ileal y fecal de mucinas ($P \leq 0.033$). El nivel de treonina no afectó a ninguna de estas mediciones, sin embargo hubo un efecto positivo en la digestibilidad ileal del almidón en las dietas con bajo nivel fibra soluble ($P < 0.001$). Un mayor nivel de fibra soluble en la dieta incrementó la digestibilidad ileal y fecal de la FDT ($P \leq 0.065$), sin afectar el peso de los órganos. En conclusión, el incremento del nivel de fibra soluble mejora la integridad de la mucosa intestinal y la secreción de mucinas, mejorando el

estado sanitario de los animales cuando las condiciones sanitarias empeoraron. El nivel de treonina no afectó a ninguna característica de la barrera intestinal, si bien cuando las condiciones sanitarias son deficientes un nivel bajo de treonina deterioró el estado de salud de los animales, sugiriendo una limitación de este aminoácido.

# RESUMÉ

L’objectif de ce travail était d'étudier l'effet des fibres solubles et la carence en thréonine sur la digestion et la performance après le sevrage chez les lapins. Quatre régimes ont été utilisés dans un arrangement factoriel de 2 x 2, avec deux niveaux de fibres solubles (89 vs.119 g/kg) et deux niveaux de thréonine (5 vs. 6,4 g/kg). L'effet des fibres solubles a été étudié en substituant la paille de luzerne (100 g/kg) par la pulpe de betterave sucrière (126 g/kg), et le niveau de la thréonine a été obtenu en supplémentant la ration par la L-thréonine (0 vs 1,42 g/kg). Dans la première expérience, 128 lapereaux, de deux sexes, sevrés et de 35 jours d'âge ont été utilisés pour déterminer la digestibilité fécale apparente de MS et PB entre le 42$^{ème}$ et 46$^{ème}$ jour (12/ration), la digestibilité iléale apparente de MS et BP (au 46$^{ème}$ jour, 20/ration) et la morphologie de la muqueuse (10/ration). Dans la deuxième expérience, 140 lapereaux sevrés de 25 jours d’âge et des deux sexes ont été utilisés pour déterminer la digestibilité fécale apparente (entre 32 à 35 jours, 11/ration) et iléale (à 35 jours. 20/ration) de la MS, PB, FDT, la concentration de l'amidon et de la mucine, la morphologie de la muqueuse (10/ration) et les paramètres de croissance (35/ration). Dans les deux expériences, les lapereaux ont été nourris avec les rations expérimentales pendant 10 jours, puis ils ont été abattus. Dans la première expérience, les lapins alimentés par un niveau élevé de fibre soluble ont montré poids relatif plus élevé de l'appareil digestif total et de caecum, en plus, un pH caecal plus bas que ceux nourris avec un faible niveau de fibre soluble ($P\leq 0,032$), sans effet sur les paramètres de croissance. L'augmentation du niveau de fibres solubles dans l'alimentation a conduit à la formation de villosité plus longue y un ratio de profondeur/hauteur des cryptes supérieur ($P<0,001$) et augmenté le nombre de cellules caliciformes par villosités ($P<0,001$). Le niveau de thréonine n’a eu aucune incidence sur les paramètres. Dans la deuxième expérience, la mortalité a diminué avec le niveau élevé de la fibre soluble ($P=0,002$), et tend à être réduit avec le niveau thréonine ($P = 0,091$). L'augmentation du niveau de fibres solubles a également augmenté le rapport hauteur/profondeur des cryptes des villosités ($P<0,001$), le nombre de cellules à mucus ($P = 0,008$) et le flux iléal et fécal des mucines ($P\leq0,033$). Cependant, aucun effet du niveau de la thréonine sur ces caractéristiques de croissance a été détectée, mais un effet positif sur la digestibilité iléal de l'amidon dans le ration alimentaire de faible contenu en fibres solubles a été trouvé ($P<0,001$). La digestibilité iléale et fécale de FDT augmentait avec

le niveau de fibres solubles (P≤0,065), sans effet sur le poids des organes. En conclusion, l'augmentation de fibres solubles améliore la sécrétion de mucus intestinal et l'intégrité des muqueuses conduisant à un meilleur état de santé des lapins quand les conditions sanitaires sont détériorées. Les caractères de la barrière muqueuse n'ont pas été affectés par le niveau de thréonine alimentaire bien que dans les conditions sanitaires déplorables, un faible niveau thréonine altère l'état de santé suggérant un effet limitant de cette acide aminé.

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# I. INTRODUCTION AND OBJETIVES

Recent studies have been focused on optimizing the post-weaning feed to improve digestive health in weaning rabbits. Total dietary fibre is the main component in the feed of rabbits accounting for 35 and 50% of the diet. Its importance is related to the influence exerted on the rate of passage of digesta and its function as substrate for microbiota, which in turn affect and regulate rabbit growth performance and digestive health. Insoluble fibre is the most important total dietary fibre fraction. It has been extensively studied and their chemical and physical characteristics play an important role in the digestive physiology of the rabbit and it is essential to avoid digestive disturbances (Gidenne et al., 2010).

In contrast, soluble fibre has been scarcely studied in rabbit nutrition. It comprises non-starch and non-NDF polysaccharides (Hall, 2003). It is a minor, heterogeneous and highly degradable fraction of the total dietary fibre (Trocino et al., 2013a). Most diets in rabbits include sugar beet pulp (SBP) to increase the level of soluble fibre. The level of soluble fibre recommended is around 12% in the diet in the growth period (Trocino et al., 2013a). In a context of epizootic rabbit enteropathy, the inclusion of soluble fibre in the diet has been shown to have positive effects on intestinal health, which has been associated to an improvement of the intestinal mucosa integrity and the modulation of intestinal microbiota (Gómez-Conde et al., 2007, 2009). Another important effect of the inclusion of soluble fibre in the diet is its positive effect on mucin secretion. Studies carried out in rats by Satchithanandam et al. (1990) found that supplementation with 5% citrus fibre in a purified diet increased the mucin secretion in the stomach, small intestine and colon. In rabbits, the inclusion of sugar beet pulp and pectin as source of soluble fibre increase the number of goblet cells, the mucin concentration in the ileum and mucin flow to the caecum (Abad, 2011; El Abed et al., 2011b).

Mucins are important components of the mucus layer. They are secreted by globet cells and play an important role in the prevention of chemical, mechanical, enzymatic damage of intestinal mucosa and bacterial adhesion in the mucosa (Deplancke and Gaskins, 2001). Mucins contain on average 20% of protein that is particularly rich in

threonine, serine and proline, representing 42% of amino acids composition of intestinal mucin in rabbits (Roberton et al., 1989). The increase of mucin production might modify the threonine requirements. The aim of this work was to study the effect of soluble fibre and threonine deficient diets on rabbits digestion and performance. To prove this effect four diets were formulated combining 2 levels of soluble fibre and threonine (according to requirements vs. deficient) (de Blas and Mateos, 2010; Trocino et al., 2013a)

**OBJETIVES:**

Identify the effect of soluble fibre, threonine and the possible interaction between the level of soluble fibre and threonine on:

- Growth performance in the post weaning period.
- Digestibility of nutrients (ileal and faecal digestibility).
- Mucin production.
- Morphology of the intestinal mucosa (villus height, crypt depth and goblet cells number).

# II. LITERATURE REVIEW

## 2.1. Definition of dietary fibre

Dietary fibre (DF) has been defined as the "sum of lignin and all polysaccharides that are not digested by enzymes secreted by the digestive system of man" (Trowell et al., 1976) and chemically defined as the set of non-starch polysaccharides and lignin that belong to the cell wall (Theander et al., 1997). It has been difficult to establish a precise definition of dietary fibre due to complex physical structure, chemical composition and high number of constituents. Recently, the Codex Alimentarius Commission (2009) defined dietary fibre as the carbohydrate polymers with ten or more monomeric units, lignin and other associated constituents, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans and exert some specific physiology effects in the digestive tract.

## 2.2. Characterization and quantification of dietary fibre

In general, dietary fibre is constituted by a series of components distributed in the plant cell wall and in the cytoplasm, according to a chemical structure that results in specific physical properties (Gidenne et al., 2010. See Figure 1). Plant cell wall is composed of microfibrils of cellulose forming a strong framework that gives rigidity to the plant. The microfibrils are embedded in a matrix composed by a lignin network cemented with another matrix of polysaccharides and glycoproteins (Cápita, 1996). Other polymers that comprise the cell wall are hemicellulose and pectin substances. The main cytoplasm components are storage carbohydrates like fructan, mannans, oligosaccharides and resistant starch and their proportion, as other components, depends on the particular plant. Full details of the fibre components of the main raw materials used in animal feed are described by Selvendran (1984), Bach Knudsen (1997), Gidenne et al. (2010) and Hall (2003). The most common definitions to refer to the animal physiology are insoluble and soluble fibre. The first one is comprised mainly by cellulose hemicelluloses and lignin, represents the greatest proportion of total dietary fibre (TDF). Soluble fibre (SF) represents the lowest proportion of TDF, that comprises the non-starch and non-NDF polysaccharides, the main components that are considered

part of the soluble fibre fraction are pectin substances, (1 → 3) (1 → 4)-β-glucans, fructans and gums (Hall, 2003).

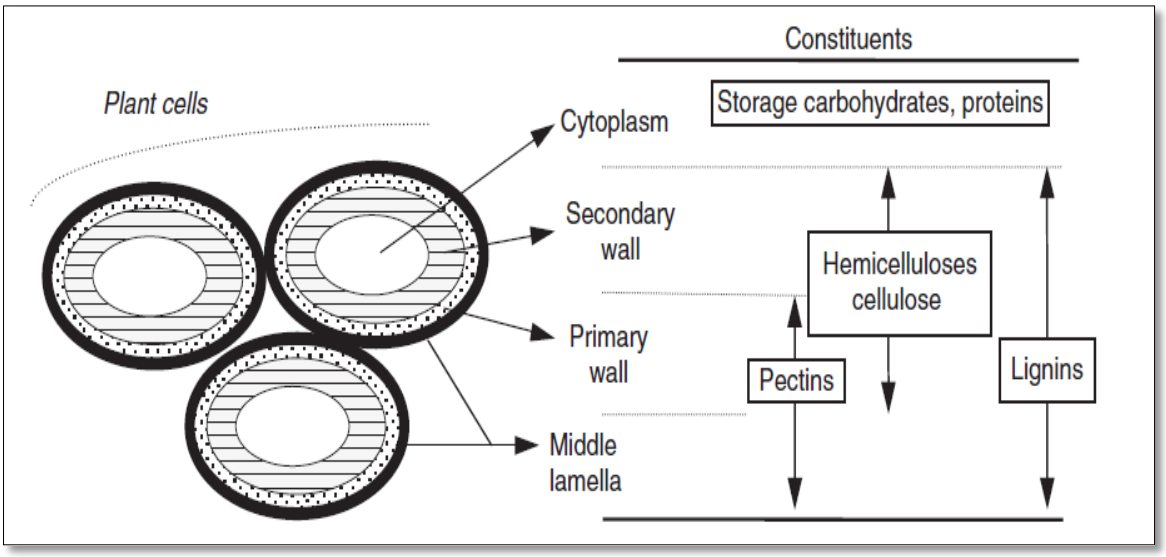


**Figure 1: Schematic representation of the cell wall of the plant and its main components. (Gidenne et al., 2010).**

Due to the heterogeneity in the three-dimensional matrix of plant cell walls there is not available a methodology to accurately determine the dietary fibre of a feedstuff. According to the concept of dietary fibre, it could only be determined by the digestive balance in the animal. An indirect estimation of dietary fibre may be performed by different methodologies reviewed by (Bach Knudsen) 2001 and Mertens (2003) in which non-fibrous constituents are extracted well solubilized with chemical solutions, hydrolyzed enzymatically or by combining both procedures, once isolated the residue of fibre can be measured gravimetrically or chemically, leading to three methods: chemical-gravimetric, enzymatic-gravimetric and enzymatic-chemical methods.

The most widely method used for determining TDF is the enzymatic gravimetric, this method simulate the digestion when incubating the samples with enzymes (amylases, proteases and amyloglucosidase). The TDF can divide into soluble (SDF) e insoluble fibre (IDF). Ethanol is used to precipitate and recover the indigestible water soluble polysaccharides (Prosky et al., 1992; AOAC, 2000). An alternative measure of soluble fiber is that of neutral detergent-soluble fiber, or those non-starch non-NDF polysaccharides soluble in neutral detergent plus heat-stable, α-amylase. (Hall et al., 1997, 1999).

In order to determine the component of cell wall matrix and estimate its major subcomponents (cellulose, hemicelluloses and lignin) the sequential analyses of detergent system: neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were developed by Van Soest (1963). Modifications with heat stable α-amylase (aNDF) have been proposed to extent this application to grains, concentrated feed, also this fraction has been corrected by ash and reported in organic matter (aNDFom) (Mertens et al., 2002). In order to compare with another method for insoluble fibre, this fraction also need to be corrected for crude protein (CP) (aNDFom - cp) (Abad et al., 2013).

Another alternative to estimate insoluble fibre is the in vitro indigestibility dry matter (ivDMi). Ramos et al. (1992) adapted the enzymatic in vitro method developed by Boisen (1991) for pigs to estimate the nutritive value of rabbit feeds. The aim of this method is to remove the digestible fractions of diet by using enzymes in three steps, the main part of indigestible fraction of the diet is dietary fibre. In order to compare with another method for insoluble fibre, the ivDMi2 and 3 steps need to be corrected for ash and crude protein (Abad et al., 2013).

Soluble fibre can be estimated by difference using three procedures: TDF–IDF ($SDF_{IDF}$), TDF–ivDMi2 ($SDFiv_{DMi2}$), and TDF – aNDFom-cp ($SDF_{aNDFom-cp}$). (Abad et al., 2013). The quantification of soluble fibre depends on the method used to determine it, showing $SDF_{aNDFom-cp}$ the highest value between the three procedures (Abad et al., 2013). This method is used in most studies related on the role of soluble fibre in growing rabbits (Trocino et al., 2013a). The differences observed between these three procedures might be accounted for the different analytical conditions used in extraction procedures (mainly temperature, but also pH and reagents) to remove starch and protein in each method (Marlett et al., 1989). This is especially important in pectin rich feedstuffs, like SBP, where the use of boiling NDF solution (containing EDTA, a chelating agent of calcium bound in pectin complexes) led to solubilization of a higher amount of substances than for ivDMi2. While the use of $SDF_{IDF}$ showed intermediate values between $SDF_{aNDFom-cp}$ and $SDF_{ivDMi2}$ (Abad et al., 2013).

Dietary fibre the major fraction of rabbit diets, where it accounts for 40 – 50% of the total diet. The levels of fibre in complete experimental feeds used for the growing rabbit are shown in Table 1.

**Table 1: Levels of fibre (g kg$^{-1}$ dry matter) in complete experimental feeds used for the growing rabbit (n = 111) (Villamide et al., 2009).**

| | Average | Minimum | Maximum |
|---|---|---|---|
| aNDFom | 368 | 248 | 443 |
| ADFom | 169 | 135 | 284 |
| ADL | 56 | 27 | 195 |
| Hemicellulose | 172 | 59 | 251 |
| Cellulose | 140 | 42 | 220 |
| *SF | 77 | 18 | 147 |
| Other feed constituents | | | |
| Starch | 176 | 82 | 324 |
| Crude protein | 166 | 122 | 244 |
| Sugars | 53 | 31 | 163 |
| Ether extract | 32 | 10 | 71 |

aNDFom: neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; ADF: acid detergent fibre; ADL: acid detergent lignin; CP: crude protein; EE: ether extract.
*SF: determined by (Trocino et al., 2013a) using 18 experimental diets, the soluble fibre was calculated by difference between TDF–NDF, or as neutral detergent soluble fibre (NDSF, Hall et al., 1997), the pectin content of the diets given in the paper.

### 2.3. Effect of dietary fibre on digestion and gut barrier health

#### 2.3.1. Fibre digestion

Dietary fibre can be digested only through microbial fermentation in the digestive tract (de Blas et al. 1999). Some components of dietary fibre are degraded prior to entering the caecum of rabbit by the pectinase activity, the main fibrolytic enzyme present in the stomach, small intestine and cecum, and it is explained by caecotrophy (Marounek et al. 1995). One of the most important constituents of the pectins are uronic acid, that showed a relative high ileal digestibility (20 to 52%) (Gidenne, 1992; Carabaño et al., 2001). Moreover the high digestibility of soluble fibre in the ileum can be explained by the existence of a wide type of ileal microbiota (Gómez-Conde et al., 2007, 2009). On the other hand, glucose and xylose the major monomers from insoluble fibre in most fibre sources, showed a much lower ileal digestibility (Gidenne, 1992; Carabaño et al. 2001). Ileal digestibility coefficient of aNDFom-cp and SF (TDF – aNDFom-cp) increased with the level of soluble fibre, with increasing the proportions of SBP and apple pulp (Abad et al., 2012). Despite the low digestibility of the insoluble fibre, supplying insoluble dietary fibre to growing rabbits is essential to avoid digestive disturbances, as it warrants an adequate rate of passage (Gidenne et al., 2010).

The main site for dietary fibre fermentation seem to be the caecum, because of the higher microbial activity of the digesta and longer retention time in this segment, although it is mainly fermented the soluble fibre and short sized fibre particles (Gidenne et al., 2010). The ileal and caecal microbial population secretes enzymes capable of hydrolyzing the main components of dietary fibre. Greater enzymatic activity for degrading pectins and hemicelluloses than for degrading cellulose has been detected in several studies (Marounek et al., 1995; Gidenne et al., 2000, 2002). The source of fibre has a significant effect on the enzymatic activity (pectinolytic and cellulolytic) being higher in SBP diets than in alfalfa and wheat bran diets (Falcao e Cunha et al., 2004). It agrees with the increases in the digestion efficiency of fibre and in the microbial activity with the level of pectin substances in the diet Garcíaet al. (2000), such as accurs when SBP is introduced in them (Trocino et al., 2013a). These results are parallel to the faecal digestibility of the corresponding dietary fibre constituents in rabbits. Hemicelluloses show a higher digestibility than cellulose (46 vs. 27%). (Gidenne et al., 2010). Although the relative contents of digestible hemicellulose and cellulose in the diet might vary depending on the source of the fibre used (Trocino et al., 2013a). Lignin and cutin are considered almost totally undegradable and might limit the digestibility of hemicellulose in a great extent than that of cellulose (Gidenne et al., 2010). Therefore, increasing the levels of sugar beet pulp as source of soluble fibre with low lignified (insoluble fibre) and with a high hemicellulose to cellulose ratio has often been associated with an increase NDF faecal digestibility (See Figure 2), improving digestibility and energy value. In recent studies the apparent faecal digestibility of soluble fibre has been determined showing a range from 69.7 to 95.1% and a mean of 84.9%. (Abad. 2011, 2012; Trocino et al., 2010, 2011, 2013b). In all the cases the soluble fibre was determined by the difference between (TDF - aNDFom-cp).

The inclusion of SBP as source soluble fibre in the diets, is associated with an increase of the relative weight of total digestive tract, stomach, caecum, as well as an increase of the volatile fatty acid concentration, especially acetate and propionate, and a reduction of caecal pH (Fraga et al., 1991; García et al., 1993; Carabaño et al., 1997; García et al., 2002b; Falcao e Cunha et al., 2004: Gómez-Conde et al., 2009; Trocino et al., 2011). This effect might be due to insoluble fraction of SBP rather than to the soluble fraction (El Abed et al., 2011a).

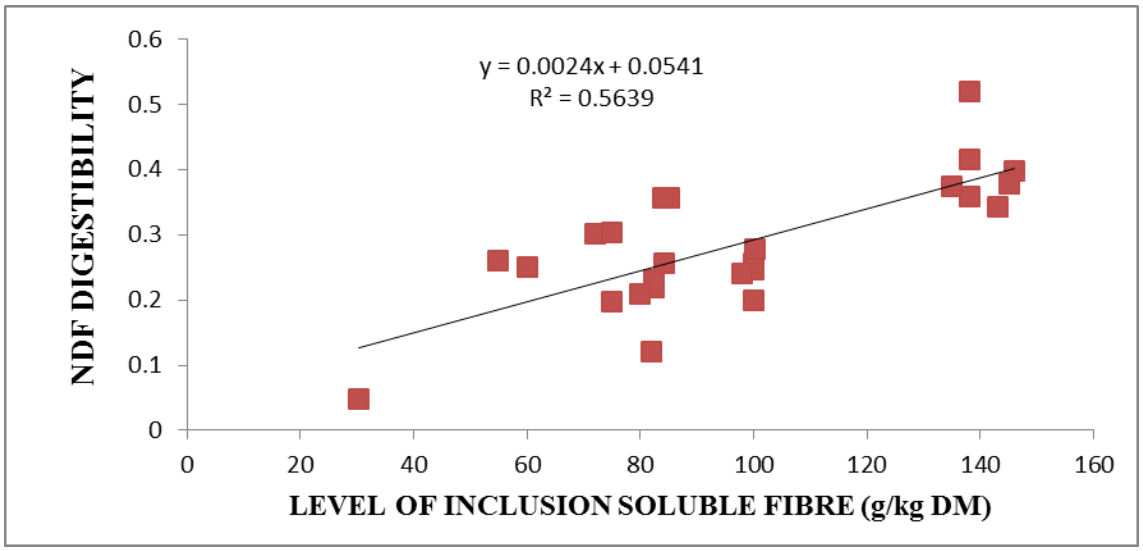


**Figure 2: Effect of level of inclusion of soluble fibre on NDF faecal digestibility (Abad et al., 2011, 2012; Trocino et al., 2010, 2011, 2013b).**

### 2.3.2. Effect of dietary fibre on gut barrier health

The intestinal barrier plays a key role in the protection of rabbits and other animals against pathogens because it prevents the colonization and the translocation of bacteria and toxins. (Carabaño et al., 2008). Intestinal barrier begins in the intestinal lumen through the acidification and continues with the protection mechanisms of the epithelium by a mucus layer secreted by goblet and paneth cells. This mucus protects the lining of mechanical chemical or enzymatic damage and bacterial adhesion (Deplancke and Gaskins, 2001). Once this protection is lost and bacteria and toxins are in contact with the epithelium, the mucosal immune system linked to the mucosa is put into operation, first non-specifically and then developing tolerance mechanisms (Montage et al., 2004). Several criteria have been proposed to characterize mucosal integrity in poultry and pigs. Some of the most frequently used parameters are: enzyme activity in the lumen, brush border enzyme activity, mucosa morphology, others barrier function trait and microbial activity (Van der Kils and Jansman, 2002).

In non-ruminants, level and source of dietary fibre affect intestinal mucosa (Montagne et al., 2003). In rabbits, both insoluble and soluble fibre plays an important role in morphology of intestinal mucosa. The inclusion of soluble fibre in the diet in substitution of insoluble fibre, improves the structure (villous height/crypt depth) and functionality (greatest sucrose activities) of mucosa and the immune response (See

Figure 3, Gómez-Conde et al., 2007). This study suggested that the soluble fibre has a protective effect upon the mucosa that favors an immune response. This positive effect of soluble fibre on the intestinal mucosa may also be related with the modulation of caecal microbiota (Gómez-Conde et al., 2007, 2009), reducing the frequency of detection of several potential pathogens as *Clostridium perfringens* and *Campylobacter spp*. These results suggest a positive influence of soluble fibre on intestinal health. Likewise pectin compared highly lignified fibre improved morphology of the jejunal mucosa and the activity of intestinal cells (Chiou et al., 1994). Similarly, rabbits fed with SBP showed higher villi height / crypt depth ratio than those fed with sunflowers hulls and straw (El Abed et al., 2011b). These authors also found that the animals fed with the soluble fraction of SBP (pectin) or with the insoluble fraction of SBP, showed intermediate values between sunflowers hulls/straw and SBP diet. Therefore, it seems that the beneficial effect on intestinal morphology is an additive effect of both soluble and insoluble fibre fractions of the SBP. However, these changes in gut mucosa morphology might be age-dependent as they were lower in animals at 45 d of age (Àlvarez et al., 2007) or not observed in older rabbits (51-56 d of age) (Trocino et al., 2010, 2011; Xiccato et al., 2011). The sampling site (jejunum vs. ileum), time after weaning and health status of the animals may also contribute to these differences (Trocino et al., 2013a).

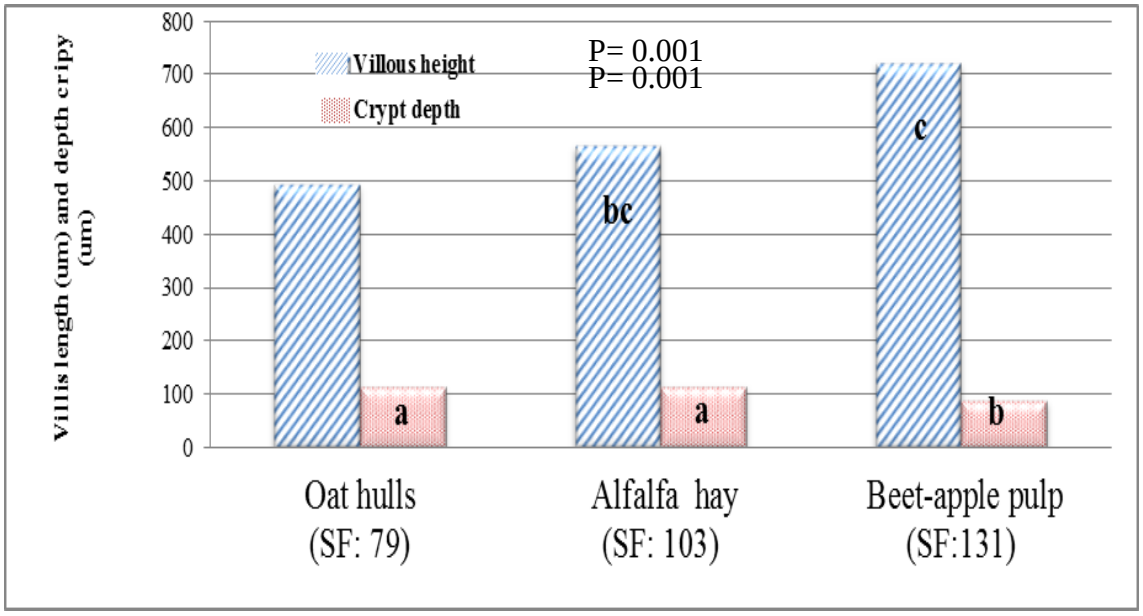


**Figure 3: Effect of fibre source and soluble fibre levels on the morphology of the jejunal mucosa in 35-d-old rabbits (Gómez-Conde et al., 2007).**

**a**

The effect of insoluble fibre on mucosa morphology in rabbits might depend on the level insoluble fibre (Gutiérrez et al., 2002; Yu and Chiou, 1996; Chao and Li., 2008) and on the type of fibre (Chiou et al., 1994; García et al., 2002a; Alvarez et al., 2007) but the results vary widely probably due to the different sources of fibre used.

The inclusion of pectin in diet for rats also increase the villus height and crypt depth, thus suggesting that it led to a mucosa hyperplasia in the small intestine, which might be mediated through the generation of short-chain fatty acids (Pirman et al., 2007). In contrast the inclusion of pectin in weaning diets for pigs decreased the villous height and crypt depth and the area of mucins in the crypts of the small intestine, whereas the feeding with high insoluble fibre diets improved gut morphology by increasing villi length and increased mucosal enzymatic activity (Hedemann et al., 2006). These results evidence the different effect of soluble fibre depending on the animal, which might be related to the differences in the intestinal physiology and microbiota

As mentioned above intestinal mucus plays an important role in the prevention of damage in the mucosa. To evaluate the ability of both crude mucus and mucin, to inhibit mucosal adherence of enteric pathogens, Drumm et al. (1988) examined whether mucus and mucin derived from rabbit ileum interact with the rabbit enteropathogen *Escherichia coli* RDEC-1. Data obtained indicated that the *Escherichia coli* enteropathogen RDEC-1 can bind to purified glycoproteins of goblet cell origin and that adherence of these bacteria to mucin is mediated by expression of pili, avoiding the attachment to the villi.

The mucus layer is composed primarily of water (95%), but also contains salts, lipids such as fatty acids, phospholipids and cholesterol and proteins with a defensive purpose such as lysozyme, immunoglobulins, growth factors and trefoil factors. The main component that is responsible for its viscous and elastic gel-like properties is the glycoprotein mucins, which are secreted by globet cells (Allen et al., 1982). Mucins are classified into neutral and acidic subtypes, the latter are further distinguished by sulfated

(sulfomucins) and not sulfated (sialomucins) group, the neutral mucins appear to be predominant subtype expressed in gastric mucosa and the acidic mucins are expressed throughout the intestinal epithelium and dominate in the large intestine (Deplancke and Gaskins, 2001). Changes in goblet cell function and in the chemical composition of the intestinal mucus have been detected in response to a broad range of luminal insults, including changes in the normal microbiota and the intrusion of harmful enteric pathogens (Deplancke and Gaskins, 2001; Bansil and Turner, 2006; McGuckin et al., 2011 ). Studies carried out in rats suggest that the acidic mucins protect against bacterial translocation because sulfated mucins appear less degradable by bacterial mucolytic activities and host proteases (Rhodes, 1989). In rabbits there is scare information about mucin production dynamics, nevertheless it has been observed that rabbits affected by epizootic enteropathy present large amount of mucus secreted by the small intestine as a reply to the disease (Licois et al., 2005).

Mucin secretion also can be influenced by the diet. The effect of fibre on mucin secretion seems depend on fibre solubility. In pig the inclusion of insoluble fibre diet enhanced the mucus secretion (See Figure. 5) (Montagne et al., 2004). Meanwhile, the relationship between intestinal mucin secretion and soluble dietary fibre has not been fully elucidated. Satchithanandam et al. (1990) found in rats that supplementation with 5% citrus fibre in a purified diet produced an increase of mucin secretion in the stomach, small intestine and colon. According to Ito et al. (2009) the secretion in the jejunum and ileum luminal mucin content and goblet cell number in the jejunum and ileum increased in proportion to the molecular weight of soluble fibre source. In pigs, using a highly viscous non-fermentable soluble polysaccharide, carboxymethylcellulose showed an increase of the number of ileal goblet cells and luminal crude mucin (Piel et al., 2005). These observations may suggest that soluble fibre viscosity might be a contributing factor in small intestinal mucin secretion.

The inclusion of SBP in rabbit diets increased the mucin concentration in the ileal digesta and the mucin flow to the caecum. This effect seems on be more dependent of the soluble fraction of the SBP, than on the insoluble one (Abad, 2011). The soluble fraction of SBP enhanced the number of goblet cell per villi in the jejunum and the ileal flow of mucin (El Abed et al., 2011b). The mucins that reach to the caecum are apparently totally fermented (from 93 to 96%) (Abad, 2011).

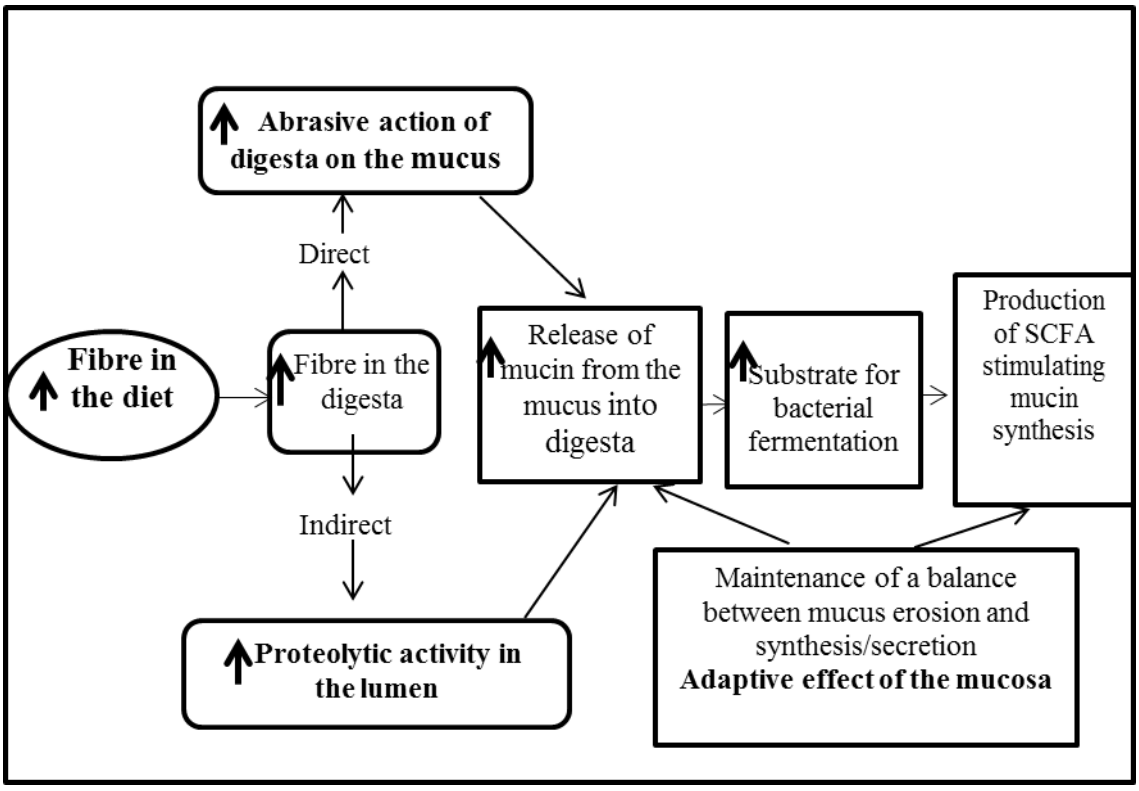


**Figure 4: Hypothetical effects of dietary fibre on the balance between mucus erosion releasing mucin into the gut lumen and synthesis (Montage et al., 2004).**

## 2.4. Effect of dietary protein on digestion and gut barrier health

### 2.4.1. Protein digestion

Protein digestion takes place through proteolytic enzyme activity. This enzyme activity is distributed in the stomach, small intestine, caecum and colon (Marounek et al., 1995). Research conducted by Dojana et al. (1998) using rabbits from 15 to 180 day of old, reported that pepsin activities were relatively constant in both young and adult rabbits, while trypsin and chymotrypsin reached a peak at about weaning and then remained constants. Debray et al. (2003), indicated that between 25 and 42 days of age, pancreatic specific activities of trypsin and chymotrypsin did not change. However, total activities and relative activities expressed on a live weight basis were increased after weaning (63% trypsin and 56% chymotrypsin) mainly due to the specific increase of the organ weight and pancreatic protein content. Additionally, in the same study when the activities of pancreatic enzymes were measured in the whole small intestinal

contents, it increased during the same period but the range of variations was lower than those measured in the pancreatic gland.

Despite the discrepancies in proteolytic enzyme activity the apparent ileal digestibility of crude protein in young rabbits (21 and 35-d old) was similar or even higher compared to that of older animals (42 and 45-d old), using different sources of protein (Garcia-Ruiz et al., 2006; Gallois et al., 2008). These results might be partially explained by a relatively lower ileal flow of endogenous nitrogen in young compared to older rabbits. This fraction is an important proportion of the apparent ileal flow in rabbits and are highly dependent on feed intake (García et al., 2004), which in turn increases sharply after weaning (Gómez-Conde et al., 2011).

Once the digesta reaches the caecum the caecal microbiota has an important role in the digestion of undigested protein and endogenous protein as it may convert non protein nitrogen into microbial protein (Yoshida et al., 1968). The efficacy of synthesis of microbial protein depends on the ammonia and energy availability (Carabaño et al., 2009). As reviewed by Fraga (1998), the caecal ammonia concentration is negatively correlated to the dietary digestible energy (DE): digestible protein (DP) ratio. When, the protein intake exceeds nutritional requirements, urea recycling from the blood to the caecum increase, leading to an elevation caecal ammonia concentration.

Apart from microbial protein, another end product of microbial fermentation in the caecum are volatile fatty acids. Its concentration depends on the level and type of dietary fibre. It increased with the level of dietary NDF and uronic acids, the major constituents of pectin and decreased with the degree of lignification of NDF (García et al., 2002b). The proportion of microbial protein with respect to total protein in soft faeces ranges from 40 to 67% (García et al., 2000; García et al., 2005). Soft faeces intake accounts for 7 to 20% to the total dry matter intake (DMI), while crude protein accounts from 11 to 24% to the total protein intake (See Figure 5).

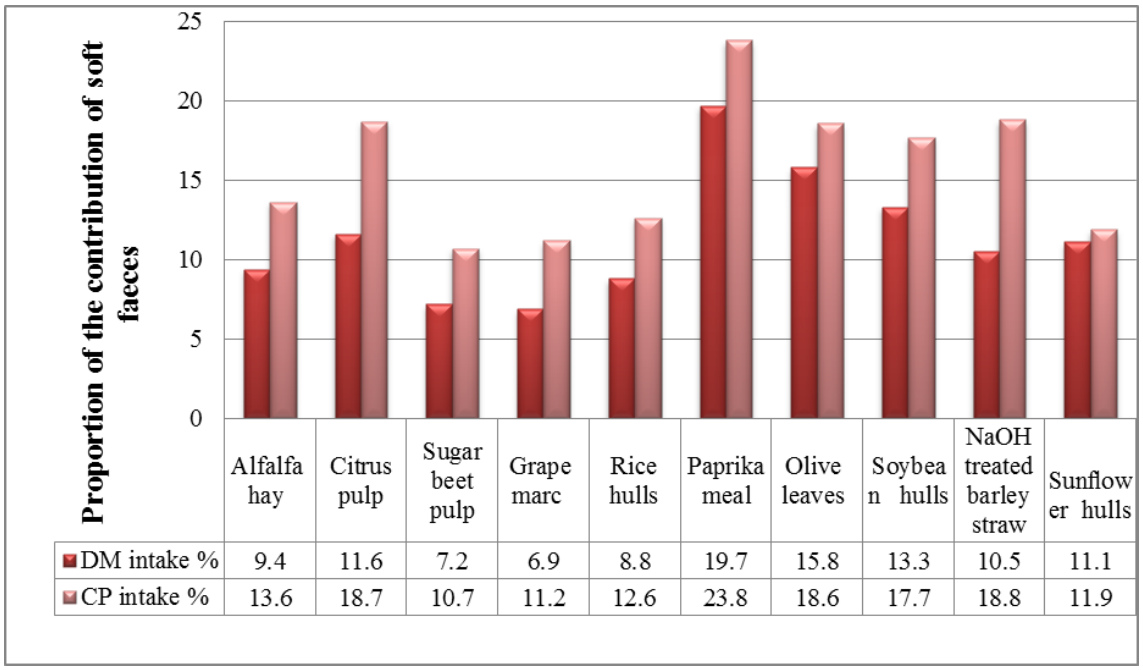


**Figure 5: Contribution of soft faeces to the total DMI and crude protein intake, g/d (Fraga et al., 1991; Garcíaet al., 2000).**

**Table 2: Amino acid composition and relative contribution of soft faeces to the total intake of amino acids, g / d (Nicodemus et al., 1999¸García et al., 2004)**

| | Soft faeces amino acid composition (g/16g N) García et al., (2004) | Soft faeces amino acid contribution (%) to DMI Nicodemus et al., (1999) |
|---|---|---|
| Essential amino acids | | |
| Arginine | 4.42 | 8 |
| Cystine | 2.28 | 14 |
| Histidine | 1.38 | 10 |
| Isoleucine | 3.47 | 16 |
| Leucine | 5.01 | 13 |
| Lysine | 4.11 | 18 |
| Methionine | 1.16 | 17 |
| Phenylalanine | 2.49 | 13 |
| **Threonine** | **5.60** | **21** |
| Valine | 5.33 | 17 |
| Nonessential amino acids | | |
| Alanine | 4.18 | 19 |
| Aspartic acid | 8.20 | 17 |
| Glutamic acid | 9.81 | 11 |
| Glycine | 4.38 | 15 |
| Proline | 3.68 | - |
| Serine | 4.28 | 16 |
| Tyrosine | 2.44 | - |

The final result of bacterial activity in the caecum is a substantial change in the amino acid composition of the protein that enters to the caecum from the ileum (Table 2). According to Garcíaet al. (2005) the bacterial activity leads to enrichment in lysine (0.072 g/d), methionine (0.06 g/d) and threonine (0.059 g/d). As consequence the ingestion of soft faeces enables to digest microbial protein and to use part these amino acids (Villamide et al., 2010). The contribution of soft faeces to the total intake of amino acids was 17%, 18% and 21%, for the essential amino acid methionine + cysteine, lysine and threonine, respectively (Table 2; Nicodemus et al., 1999). Early studies, carried out by Davidson and Spreadbury (1975) indicate that amino acid balance should be less critical in rabbit than others non-ruminants.

### 2.4.2. Effect of protein on gut barrier health

In the first weeks of rabbits life, the intestine is the portion of the gastrointestinal tract with the highest growth (Lebas and Laplace, 1972). Specifically the intestinal mucosa has high requirements of amino acids. In the case of piglets the intestinal mucosa utilizes considerable amounts (40–60%) of dietary amino acids (Stoll et al., 1998). Therefore the level and type of protein could affect the small intestine mucosal integrity especially in young rabbits, however there is scarce information. In rabbits, weaning involves a change of the level and type of protein; the milk easily digestible proteins are changed by vegetable proteins of worse digestion and containing anti-nutritional factors that can damage the intestinal mucosa (Gutiérrez et al., 2002). The substitution of vegetable proteins like soybean meal with a highly digestible protein like animal plasma had a positive effect on the morphology of intestinal mucosa (Gutiérrez et al., 2000). Several studies in pigs suggested that animal plasma may protect against the development of mucosa damage and thus should allow less passage of inert large molecules through the intestinal wall. The immunoglobulins and glycoprotein fractions of animal plasma might prevent attachment of pathogens and thus support functionality of the intestine (Van Dijk et al., 2001). However, the use of using different sources of protein (soya bean meal 48, soya bean protein concentrate, sunflower meal 36 and a combination of soya bean meal 48 and potato protein concentrate) in early-weaned rabbits, did not affect the jejunum morphology (Gutiérrez et al., 2003). The substitution

of alfalfa hay with soya-bean protein concentrate in starter diets for young rabbits did not affect jejunal morphology (Chamorro et al., 2007).

There is scarce information about the effect of the protein on the mucus layer especially in rabbits. Studies performed in calves showed that both the level and source of protein influenced the production of mucin. When dietary crude protein supplied by skim milk powder rose from 14 to 278 g of CP/kg DMI, the flow of mucin protein increased at the duodenum (+300%) (Montage et al., 2000). The flow of mucin protein also, increased by 70% at the duodenum and at the jejunum when protein from skim milk powder was partially replaced by soybean protein concentrate and hydrolyzed soybean protein isolate. When it is replaced by potato protein concentrate, the mucin flow also increased at the duodenum (+24%) and at the ileum (+52%) (Montage et al., 2000). In pigs the study conducted by Piel et al. (2007) showed that both protein and fibre types affected mucin secretion. Diets with high indigestible protein and fibre increased mucin secretion by 46% in relation to other more digestible diets.

### 2.5. Importance of nitrogen endogenous losses in rabbit

The classical definition of endogenous nitrogenous comes from Mitchell (1924), according to his definition, endogenous nitrogen is the nitrogen found in chyme or faeces when a nitrogen-free diet has been fed. Endogenous secretions come from various sources including saliva, pancreatic secretions, and bile, sloughed off epithelial cells, serum albumin and mucin (Nyachoti et al., 1996). In pigs, this fraction when passing into the small intestine can be hydrolyzed and absorbed before reaching terminal ileum while into large intestine can be metabolized by local microbes like the mucin, or excreted through feces (Souffrant et al., 1993). Endogenous losses have been divided into basal and the specific endogenous losses, the first one are directly related to the dry matter intake (Hess and Sève, 1999), and the specific endogenous losses are induced by specific composition and characteristics of the diet (Boisen and Moughan, 1996). In this sense, García et al. (2004) and Villamide et al. (2013) found a lineal relationship between feed intake and endogenous ileal protein flow, in rabbits using diets with highly digestibility casein. The endogenous ileal flow of N increased with the feed intake around 6 mg of N/g DMI (Villamide et al., 2013).

Endogenous nitrogen accounts for 50 -75% of the total ileal flow of nitrogen in the digesta of rabbits, the remaining percentage correspond to the indigestible nitrogen linked to NDF and to the small presence microbial nitrogen (García et al., 2005; Villamide et al., 2013). According to these studies the level of endogenous nitrogen losses in rabbits is greater to that found in pigs (5.45 g/kg DMI vs. 2.02 g/kg DMI) (Jansman et al., 2002; Villamide et al., 2013) using the same methodology of determination (casein based diet). The major reason could be the higher amount of fibre in rabbit diets respect to other non-ruminant species, 270-330 vs. 30-80 g NDF/kg DM in rabbits and pigs diets, respectively (García et al., 2005). The endogenous flow of amino acids determined from rabbits fed a casein basal diet acids varied from 5.053 g/kg DMI for glutamic acid to 0.38 g/kg DMI for methionine, being 2.21 g/kg DMI for threonine (García et al., 2004. Table 3).

**Table 3: The amino acid composition (g/16 g N) of endogenous ileal protein of rabbits fed a casein basal diet.**

| | Amino acid composition (g/16gN) | | Endogenous flow of amino acid (g/kg DMI) | |
|---|---|---|---|---|
| | García et al. (2004) | Llorente et al. (2006) | García et al. (2004) | Villamide et al. (2013) |
| Essential amino acids | | | | |
| Arginine | 4.63 | 3.60 | 1.85 | 1.23 |
| Cystine | 3.11 | 2.70 | 1.24 | 0.94 |
| Histidine | 1.53 | 1.30 | 0.61 | 0.44 |
| Isoleucine | 3.72 | 3.80 | 1.49 | 1.30 |
| Leucine | 4.77 | 4.30 | 1.90 | 1.47 |
| Lysine | 3.76 | 3.60 | 1.50 | 1.21 |
| Methionine | 0.96 | 0.70 | 0.38 | 0.27 |
| Phenylalanine | 2.09 | 4.10 | 0.83 | 1.40 |
| Threonine | 5.53 | 5.60 | 2.21 | 1.89 |
| Valine | 5.64 | 5.10 | 2.25 | 1.72 |
| Nonessential amino acids | | | | |
| Alanine | 3.56 | 3.40 | 1.41 | 1.15 |
| Aspartic acid | 7.53 | 7.20 | 3.01 | 2.46 |
| Glutamic acid | 12.7 | 12.5 | 5.05 | 4.24 |
| Glycine | 5.18 | 8.00 | 2.07 | 2.76 |
| Proline | 5.41 | 4.70 | 2.16 | 1.59 |
| Serine | 6.45 | 5.80 | 2.57 | 1.96 |
| Tyrosine | 2.07 | 3.50 | 0.83 | 1.18 |

The ileal endogenous N flow comes from different origins that condition the endogenous amino acid profile that seems to be rather constant. The predominant endogenous amino acids found in the ileal digesta are glutamic acid, aspartic acid,

threonine, serine and glycine. (García et al., 2004; Llorente et al., 2006. Table 3). The concentration of threonine is high in the ileal endogenous losses 5.53 g per 16 g N compared to lysine and methionine 3.76 g and 0.96 g per 16 g N, respectively. It implied a higher ileal flow of threonine compared lysine and methionine. This important flow of threonine can be explained by the mucins content that are rich in threonine (Roberton et al., 1989). The specific endogenous losses are related to the characteristics of the diet. The presence of fibre and anti-nutritional factors can double its production in pigs (Boisen and Moughan, 1996). Some studies have determined the true ileal digestibility of protein and amino acids from raw materials frequently used in rabbits feeding. These studies suggest that correction the ileal endogenous mainly affected the ileal digestibility of those amino acids in the major proportion in the endogenous substance flow, as threonine, and/or minority amino acid total amino acid content of the feedstuff (Table 4. García et al., 2005).

**Table 4: Apparent ileal (AI) and true ileal (TI) digestibility of raw material.**

| Raw Material | Autor | AID | | TID | | TID - AID | |
|---|---|---|---|---|---|---|---|
| | | CP | THR | CP | THR | CP | THR |
| Maize | Llorente et al. (2007) | 49.4 | 15.8 | 78.2 | 62.0 | 28.8 | 46.2 |
| Wheat | Llorente et al. (2007) | 67.0 | 44.0 | 89.1 | 84.3 | 22.1 | 40.3 |
| Wheat bran | García et al. (2005) | 52.9 | 43.9 | 69.8 | 74.4 | 16.9 | 30.5 |
| Barley grain | García et al. (2005) | 61.9 | 45.7 | 79.6 | 72.5 | 17.7 | 26.8 |
| Gluten feed | Llorente et al. (2007) | 65.5 | 49.8 | 78.1 | 71.5 | 12.6 | 21.7 |
| Peas | Llorente et al. (2006) | 76.1 | 63.3 | 86.3 | 84.6 | 10.2 | 21.3 |
| Alfalfa hay | García et al. (2005) | 59.0 | 56.2 | 74.2 | 75.2 | 15.2 | 19.0 |
| Sunflower 28% | Llorente et al. (2006) | 76.1 | 74.1 | 88.3 | 90.4 | 12.2 | 16.3 |
| Sunflower 38% | Llorente et al. (2006) | 80.4 | 76.3 | 89.0 | 88.8 | 8.60 | 12.5 |
| Soybean tosted | Llorente et al. (2006) | 82.3 | 76.1 | 93.7 | 87.9 | 11.4 | 11.8 |
| Sunflower 36% | García et al. (2005) | 80.7 | 73.8 | 86.1 | 84.6 | 5.40 | 10.8 |
| Soybean meal | Llorente et al. (2006) | 86.7 | 81.4 | 93.6 | 91.3 | 6.90 | 9.90 |

One of the most important constituents of endogenous losses are mucins, as mentioned in Section 2.3. Mucins are secreted by specialized cells called and paneth goblet cells and have an important role in the intestinal barrier. The most remarkable property of mucin is its ability to form a gel, a viscoelastic semisolid material that adheres to the epithelial surface and provides a physical barrier between the underlying cell surface and lumen (Bansil et al., 1995).

The mucin are glycoproteins that are constituted by oligosaccharide chains of 5–15 monomers, exhibit moderate branching and are attached to the protein core by O-glycosidic bonds to the hydroxyl side chains of serine, threonine and proline and arranged in a “bottle brush” configuration around the protein core (Bansil et al. 1995). Mantle and Thakore (1988) determined the carbohydrate profile of purified rabbit intestinal and colon mucins (Table 5). Whereas Roberton et al. (1989) using different techniques of purification of mucin in different species, determined protein content in different species around 20% of dry weight, and similar features in their amino acid profile (Table 6). The major amino acid residues (serine, threonine and proline) tend to comprise 40-50 mol/100mol. A recent study conducted by Romero et al. (2011), determined a protein concentration of 25% in crude mucin of ileal digesta in rabbits using different type of grinding of barley and dehydrated alfalfa, although these authors did not purify intestinal mucin from other proteins.

**Table 5: Carbohydrate profiles of purified rabbit intestinal and colonic mucins (mol/100mol) (Mantle and Thakore, 1988).**

| Carbohydrates | Upper small intestine | Mid small intestine | Distal small intestine | Proximal colon |
|---|---|---|---|---|
| Fucosa | 9.5 | 9.0 | 9.9 | 10.3 |
| Mannose | 2.1 | 2.0 | 1.6 | 1.8 |
| Galactose | 21.6 | 21.9 | 22.7 | 23.7 |
| N-acetylglucosamine | 21.9 | 21.1 | 21.1 | 21.1 |
| N-acetylgalactosamine | 28.4 | 29.8 | 29.7 | 20.2 |
| Sialic acid | 16.4 | 16 | 15.0 | 22.9 |
| Sulphate | 5.9 | 2.0 | 6.8 | 5.3 |

**Table 6: Amino acid composition of purified mucins (mol/100mol) (Roberton et al., 1989).**

| | Mucin of small intestine | | | | Mucin of colon mucin | |
|---|---|---|---|---|---|---|
| | Rabbit | Human | Pig | Rat | Rabbit | Human |
| Serine + Proline + Threonine | 42.3 | 45.5 | 52.3 | 48.7 | 42.3 | 55 |
| Aspactic acid + Glutamic acid | 14.8 | 13.5 | 8.8 | 13.1 | 16.3 | 12.3 |
| Glycine + Valine + Alanine | 20.9 | 17.6 | 16.6 | 14.7 | 18.8 | 15.9 |
| Leucine + Isoleusine | 8.2 | 10.5 | 7.4 | 8.3 | 8.4 | 8.2 |
| Rest of amino acid | 13.7 | 12.9 | 14.9 | 15.2 | 14.2 | 8.6 |
| Protein (% of dry weight) | 18.6 | 14 | 17.9 | 13.4 | 17.4 | — |

The carbohydrate structures found on mucin macromolecules are extraordinarily diverse, providing a vast array of potential binding sites for both commensal and pathogenic organisms. The attachment of microbes to intestinal mucins depends on many factors such as the composition and quantity of mucins, intestinal motility and rate of intestinal fluid flow. Increased mucin secretion depends on many factors one of them is the inclusion of fibre and protein in the diet, as previously commented. (Deplancke and Gaskins, 2001).

## 2.6. Requirements and function of threonine for growing rabbits

After lysine and methionine, threonine is the next most limiting amino acid in swine and poultry diets, while for rabbit diets is the second and sometimes the first (de Blas et al., 2000). The raw materials used in diets for rabbits have a strong deficit of threonine that might require to be incorporated into the diet as synthetic threonine (L-threonine) (Colin and Ghezal-Triki, 2001). The quantitative essential amino acid requirement of the growing rabbit, estimated, using weight gain as the response criterion, indicated a threonine requirement of 0.50% of the diet (Adamson and Fisher, 1973). An approach to balance the supply of amino acids is to use the concept of ideal protein. This method was used in growing rabbits by Moughan et al. (1988) and consists in supplying a dietary protein with an amino acid pattern similar to that of the amino acid of the whole body. Body the lysine concentration is 6.12 g/16gN and threonine concentration 3.94 g /16gN, accounting for 0.64 of lysine. This amino acid pattern can be regarded as an approximated ideal balance of dietary amino acids. Also, de Blas et al. (1998) supplementing a basal diet with L-threonine determined a minimal dietary concentration of 6.0 crude and 4.0 g/kg digestible threonine to maximize the performance of growing rabbits (See Table 7). A level of dietary threonine below or above (0.30% - 0.80%, in the diet respectively) of the optimum (0.50%) shows negative impact on feed intake and weight gain (Adamson and Fisher, 1973).

Studies carried out in pigs have demonstrated that 60% of dietary threonine is retained on the first-pass metabolism (Stoll et al., 1998). Once taken up by the mucosal cells, threonine may have different metabolic fates, including oxidation. Threonine is

catabolized either by threonine dehydratase to $NH4^+$ and 2-ketobutyrate, which is irreversibly converted to CO2, or by threonine dehydrogenase to form 2-amino-3-ketobutyrate, which is mainly converted to glycine and acetyl-coenzyme A (Ballevre et al., 1990). It appears that most threonine used by the intestine is for mucosal and secretory protein synthesis because threonine oxidation represents only 2–9% of the total threonine utilized (Schaart et al., 2005).

**Table 7: Threonine requirement expressed in units total and faecal digestible in the growing-finishing rabbits according to different authors.**

| | Thr total | Thr/lys total | Thr dig | Thr dig/Lys dig |
|---|---|---|---|---|
| Adamson and Fisher (1973) | 0.50 | 0.71 | _ | _ |
| Davidson and Spreadbury (1975) | 0.58 | 0.62 | _ | _ |
| Colin and Ghezal-Triki (2001) | 0.60 | _ | _ | _ |
| de Blas et al., (1998) | 0.60 | 0.67 | 0.40 | 0.67 |
| de Blas and Mateos (2010) | 0.62 | 0.85 | 0.43 | 0.75 |

Thr total: Threonine total, Thr/lys total: ratio threonine/lysine total; Thr dig: Digestible threonine; Thr dig/Lys dig: ratio threonine/lysine digestible.

In pigs, deficit of threonine below those recommended levels (-30%) reduced of villus height and crypt depth compared to the control diet in the ileum section whereas proximal and distal in the jejunum no differences were found, this low level of threonine did not affect growth performance nor growth of the intestine (Hamard et al. 2007). The presence of threonine is essential for the production of mucin in rat and piglets (Faure et al., 2005; Law et al., 2007). Threonine deficient diets affected negatively the quantity as well as the characteristics of mucins. This decrease in mucin production is directly related with a severely lower numbers of goblet cells (Faure et al., 2005). Other research shows, that the deficit or excess of threonine in the diet (0.37 - 1.11%, true ileal digestible threonine (TIDT) respectively) reduced the synthesis of intestinal mucosal protein and mucins compared with pigs fed 0.74 % TIDT according current NRC requirements (Wang et al., 2007). These results are consistent with later studies, which observed reduction in the levels of acidomucins and sulfomucins, respectively, in the ileum and duodenum of weanling pigs fed either a low or high level of dietary threonine (Wang et al., 2010). The mucins are continuously synthesized and there is a continuous and irreversible loss of threonine (Van Der Schoor et al., 2002). In rabbits through caecotrophy process, threonine can be recycled form microbial protein that can contribute to 21% of the total threonine intake (Nicodemus et al. 1999).

All these studies have been carried out in pigs, whereas there is scarce information on rabbits. Recent studies in pigs suggest that there may be some interaction between soluble fibre and threonine. The results of several studies (Zhu et al., 2005; Myrie et al., 2006) showed that soluble non-starch polysaccharides increased endogenous losses of amino acids especially threonine and affected the utilization of this amino acid for protein deposition and growth. However, Święch et al. (2012) showed that 8% inclusion pectin in the diet did not affect the number of goblet cell producing acidic and neutral mucins mid-jejunum and ileum. However, these authors found a the positive effect of pectin on the number of goblet cell containing neutral mucins in crypts of the ileum, that suggested an increase of the rate of mucosal protein synthesis particularly of threonine rich mucin protein. In this study the inclusion of pectin did not produce evident changes in threonine metabolism. Additionally there was a reduction of total tract protein digestibility and therefore the nitrogen retention was decreased with supplementation.

# III. MATERIAL AND METHODS

## 3.1. Experimental diets

Four diets in a 2 x 2 factorial arrangement were used with two level of soluble fibre (89 vs.119 g/kg) and two level of threonine (Thr) (5 vs. 6.4 g/kg). The effect of soluble fibre was studied substituting alfalfa hay (100 g/kg) by sugar beet pulp (126 g/kg), the level of Thr was obtained supplemented L-threonine (0 vs. 1.42 g/kg). Diets were formulated according to the concept of ideal protein, thus faecal digestible lysine levels were 0.57% in all diets in order to be a sub-limiting aminoacid according to recent lysine requirements (Carabaño et al., unpublished). In the diets supplemented with threonine the ratio digestibility threonine/lysine was 0.75, whereas diets not supplemented with threonine the ratio was 0.58. It resulted in two diets with low soluble fibre without or with Thr supplementation (LSF/LThr and LSF/HThr), and two diets with high soluble fibre without or with Thr supplementation (HSF/LThr and HSF/HThr). Diets contained similar concentrations of CP and NDF (162 and 326 g/kg DM respectively). To determine the apparent ileal digestibility, 5 g of DM/kg of alfalfa hay labeled with $Yb_2O_3$ was included in all diets. The diets were formulated to meet the nutrients requirement suggested by de Blas and Mateos (2010), except for soluble fibre and threonine. Ingredients and chemical composition of the experimental diets are showed in Table 8.

## 3.2. Experiment 1.

### 3.2.1. Animal and housing

This study was approved by the Committee of Ethics of the Departamento de Producción Animal of the Universidad Politécnica de Madrid. All animals were handled according to the principles of animal care published by Spanish Royal Decree 1201/2005 (BOE, 2005). Crossbred (New Zealand White × Californian) healthy rabbits mixed-sex were used in the experiments. The first experiment was carried out in the facilities of NUTRECO. Rabbits were housed in metabolism cages measuring 40 × 51 × 32 cm to collect their faeces in the faecal digestibility trial, while animals used to determine ileal digestibility were housed in pairs. Housing conditions were controlled during the whole experimental period as follows: a 12-h light-dark cycle was established and temperature conditions were maintained between 18 and 23°C by

heating systems combined with continues forced ventilation. Rabbits had *ad libitum* access to feed and water.

### 3.2.2. Determination of ileal and faecal digestibility

Fourty-eight mixed-sex weanling rabbits of 35-d of age and a body weight (BW) of 831 ± 90g (mean ± standard deviation) were blocked by litter and randomly assigned to the 4 experiment diets (12 rabbits/diet). After 7-d of adaptation to the diets, feed intake was recorded and the faeces collected for 3 days consecutive to determine the apparent faecal digestibility of DM and CP.

A second group of eighty weanling rabbits of both sexes, 35-d of age and a BW of 833 ± 116 g (mean ± standard deviation) were blocked by litter and randomly assigned to the four experimental diets (20 rabbits/diet) to determine the apparent ileal digestibility of DM and CP. Feed intake, growth rate, feed efficiency, mortality and morbidity were recorded up to 46-d of age. The symptoms of morbidity taken into consideration were: aqueous diarrhoea, mucus in faeces and relative low body weight as well as sharp reduction of feed intake and once slaughtered distended intestinal segments by gas and liquid and compacted caecal content. At 46-d animals with 1506 ± 171 g (mean ± standard deviation) were slaughtered by $CO_2$ inhalation between 19:00 and 21:00 h to minimize the influence of caecotrophy. The whole gastrointestinal tract was weighed, the stomach and caecum were extracted and weighed with their content and the caecal pH was measured. The caudal 20 cm of the ileum in each case was then excised, emptied and the digesta were frozen in dry ice. For the chemical analysis of the ileal content, samples were freeze-dried and grounded. Due to the small amounts of sample, ileal content from 2 rabbits for each treatment were pooled (10 pool/treatment). The ileal digestibility of DM and CP was determined by the dilution technique using ytterbium as a marker according Goméz-Conde et al. (2007).

### 3.1.4. Determination of mucosa morphology

To study mucosa morphology 6 cm sample to the measurement of intestinal mucosa morphology was excised from the middle part of the jejunum of rabbits (10/treatment) and placed into a 10% neutral buffered formaldehyde solution (pH 7.2 to 7.4).

### 3.3. Experiment 2

#### 3.3.1. Animal and housing

The second experiment was carried out in the facilities of ETS de Ingenieros Agrónomos de la Universidad Politécnica de Madrid, following the same standards as in the first experiment. The animals were caged in individual flat-deck cages measuring 60 × 25 × 32 cm high, while those used for faecal digestibility were caged in metabolism cages measuring 40 × 51 × 32 cm that allowed the faeces collection. Housing conditions were controlled during the whole experimental period as follows: a 12-h light-dark cycle was established and temperature conditions were maintained between 18 and 23°C by heating systems combined with continues forced ventilation. Rabbits had *ad libitum* access to feed and water.

#### 3.3.2. Determination of ileal and faecal digestibility

One hundred and forty weanling 25 d old rabbits of both sexes, and live weight of 355 ± 44 g (mean ± standard deviation) were blocked by litter and randomly assigned to the 4 experimental diets (35 rabbits/diet). After 7-d, feed intake was recorded and the faeces collected for 3 consecutive days to determinate from 32 to 35 d of age the apparent faecal digestibility of DM, CP, TDF and starch according to Gómez-Conde et al. (2011). Growth rate, feed efficiency, mortality and morbidity were recorded up to 35-d. Symptoms of morbidity were: aqueous diarrhoea, mucus in faeces and relative low body weight as well as sharp reduction of feed intake and once slaughtered distended intestinal segments by gas and liquid and compacted caecal content. At 35-d animals with a live weight of 764 ± 89 g (mean ± sd) were slaughtered by $CO_2$ inhalation between 19:00 and 21:00 h to minimize the influence of cecotrophy, to determine the apparent ileal digestibility of DM, CP, TDF and starch. The same measurements were made than in experiment 1 (weight of digestive organs and caecal pH) and ileal digesta was collected. Due to the small amount of sample, ileal content from 3 to 5 rabbits for each treatment were mixed resulting in 8 pool/diet, in which were determined Yb and CP to calculate ileal digestibility of DM and CP. A sample of 0.5 g from each pool was taken to constitute a unique pool of 4 g per treatment, in which were determined starch, TDF and crude mucin. The values obtained were used to calculate ileal starch and TDF digestibility in combination with the Yb content of the original pools. The ileal flow of DM, CP, starch, TDF and mucin was calculated by

multiplying the apparent ileal digestibility obtained for each pool of samples and the average daily intake of DM, CP, starch and TDF recorded for the corresponding animals.

### 3.3. Analytical methods

Procedures of the AOAC (2000) were used to determine the concentrations of DM (934.01), ash (942.05), CP (968.06), TDF (985.29) and starch (amyloglucosidase- $\alpha$-amylase method, (996.11). Dietary NDF, ADF and ADL were determined sequentially by using the filter bag system (Ankom Technology, New York, NY) according to the methods of Mertens (2002), AOAC (2000) procedure (973.187) and Van Soest et al., (1991), respectively. The total NDF content was corrected by protein and ash (aNDFom-cp). Dietary soluble dietary fibre was calculated by difference between TDF and aNDFom-cp.

Furthermore, was estimated the pepsin/pancreatin *in vitro* DM indigestibility (ivDMi2), using ankom bags according to Abad et al. (2013) to estimate a value of insoluble fibre closer to physiological conditions. The indigestible reside was also corrected for protein and ash. In addition, it was estimated the pectin, pancreatin and viscozyme (microbial enzyme complex) *in vitro* DM ingestibilibity (ivDMi3). The indigestible reside corrected by ash and protein. Ytterbium concentration of diets and ileal digesta were analyzed by atomic absorption spectrometry (Smith Hieftje 22, Thermo Jarrel Ash) (García et al., 1999). Calculations to determine apparent ileal digestibility of nutrients was performed according to Gómez-Conde et al. (2007).

To determinate mucin content were used one gram of ileal content and three grams of faeces, using the method of precipitation with ethanol as described by Leterme et al. (1998) and Romero et al. (2011) and using pectinase (Sigma P2401) to remove soluble fibre as described by Abad et al. (2013). Digestibility of TDF was corrected by the contamination of digesta TDF with intestinal mucins according to Abad et al. (2013).

The samples from jejunum were removed and washed with deionized water. They were gradually dehydrated in an ethanol series (50 to 100%), infiltrated with paraffin

wax using tissue processor LEICA ASP 300, and sectioned at 5 μm with microtome LEICA RM 2255. One slide was prepared from each sample, and contained a minimum of 4 sections separated at least 300 μm. The slides were processed for carbohydrate histochemistry using dyes. The dye method was Alcian blue reaction at pH 2.5, which stains sulfated type of acidic mucins (Kiernan, 1999), using automatic procedure with (Artisan$^{TM}$ Link Special Staining System). The sections were placed in acetic acid-AB2.5, pH 2.5, for 5 min, and then placed in alcian blue-AB2.5, pH 2.5 for 10 min, at 37ºC and subsequently washed in water for six times. The sections were counterstained with eosin, dehydrated, according to the procedure described by Brunsgaard, (1997). An average of 30 well oriented villus and crypts were selected on each stained slide for measuring their height and depth respectively (Hampson, 1986), and an average of the measurements was obtained for each animal. For each villi the amount of globet cells, with a clear positive reaction for sulfomucins was determined using a computer integrated microscope (10 and 40 × magnification, Olympus BX-40 light microscope). Images were digitally captured for later analysis using Soft software version 3.2 C4040Z (Soft Imaging System, Olympus, GmbH, Hamburg, Germany). The Figure 6, 7, 8, 9 shows the histologic sections of jejunum of the animals feed the diet L-SF/L-Thr, L-SF/H-Thr, H-SF/L-Thr, H-SF/H-Thr, respectively. These histologic sections where used to measure the villis length, crypt depth and goblet cell content sulfoacid mucin of animals in experiment 1 and 2.

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### 3.4. Statistical Analysis

The results obtained in the experiments were analysed as a completely randomised design with treatments arranged in a factorial structure 2 x 2 in which the main sources of variation were the level of soluble fibre, the level of threonine and their interaction, using the General Linear Model (GLM) procedure of SAS (Statistical Systems Institute Inc., Cary, NC). The cage was the experimental unit in the analysis of growth performance in the first experiment, while in the second experiment the rabbit was used as the eXperimental unit. Mortality and morbidity rate was analyzed using logistic regression (GENMOD procedure of SAS considering a binomial distribution) and results were transformed from the logit scale.

# IV. RESULTS

## 4.1. Experiment 1

### 4.1.1. Growth performance, health status and weight of digestive tract

In this experiment (35 to 46 d) all the animals remained in good health throughout the trial period, they adapted well to the experimental diets and neither morbidity nor mortality was observed. There was no effect of treatments on average daily gain (ADG), average daily feed intake (ADFI) and feed efficiency being on average (56.2; 102 g/d and 0.556, respectively) (Table 9).

Rabbits fed with high level of soluble fibre showed higher relative weight of total digestive tract and caecum than those fed with a low level (22.7 vs. 21.9 and 8.23 vs. 7.40 % respectively; $P<0.05$) and a lower caecal pH (5.66 to 5.50, $P=0.002$) (Table 10). The relative weight of stomach did not differ ($P>0.05$) in animals fed with the four experimental diets (6.10 % BW as average). Level of threonine did not affect any of these traits.

### 4.1.2. Determination of ileal and faecal digestibility

The ileal digestibility data the animal fed with the highest level of threonine could not be determined due to problem in the determination of ytterbium. There were no effect of treatment on the faecal and ileal digestibility coefficients of DM and CP. (Table 11).

### 4.1.3. Mucosa morphology

The increase of soluble fibre level in the diet lead to longer villous height, by 32%; ($P<0.001$) and tended to reduce the crypt depth (107.5 vs. 114 μm; $P= 0.061$) therefore showed a higher ratio villi length/crypt depth (by 39%; $P<0.001$). Likewise, the number of goblet cells per villi increased with the high level of soluble fibre by (25%; $P<0.001$) (Table 12). Level of threonine did not affect any of these measurements.

### 4.2. Experiment 2

#### 4.2.1. Grow performance and health status

Average daily feed intake (ADFI) was 13% higher from 25 to 35 d in animals fed high soluble fibre diets (52.1 vs. 58.3 g/d, P = 0.004). The average daily weight gain tended to be higher in rabbits that consumed high soluble fibre diets than in other two group (37.7 and 35.6 g/day; P= 0.10), because of their high ADFI, as their feed efficiency was similar in all treatments, although rabbits fed with high soluble fibre and high threonine tended to show a poor feed efficiency. No effect of threonine was observed on growth traits. Throughout this period, increasing the level of soluble fibre reduced mortality (15.7 vs. 2.8%, P = 0.002). While a threonine deficiency tended to increase mortality (11.4 vs. 7.1%, P = 0.091). On the other hand, morbidity was not affected by treatments (Table 13). Despite the observed effect of soluble fibre on feed intake, the treatments did not affect the weight of the digestive tract (Table 14), the stomach and the caecum (28.2, 7.51 and 9.37% BW; as average respectively; P ≥ 0.29). Caecal pH tended to decrease slightly with increasing level of soluble fibre (5.42 vs. 5.32, P = 0.10).

#### 4.2.2. Determination of ileal and faecal digestibility

There were no effect of treatment on the ileal and faecal digestibility of DM (45.2 and 66.0%; as average respectively). No treatment effect was observed in the ileal digestibility of crude protein (48.3%; as average), while the increase of threonine reduce by 2% the faecal digestibility of crude protein (81.8 vs. 80.3%, P = 0.020). In ileal digestibility of starch an interaction soluble fibre x threonine was observed due to increase of ileal digestibility of starch when low soluble fibre diet were supplemented with threonine (P <0.05). In contrast faecal starch digestibility was higher for low soluble fibre diets and lowest in HFS/HThr diet. The ileal and faecal digestibility of TDF increased with soluble fibre level (5.18 vs. 14.7% for ileal, 32.2 vs. 38.8% for faecal digestibility; P ≤ 0.065). The faecal digestibility of aNDFom-cp increased by 14% (P= 0.035) with level of soluble fibre, whereas faecal digestibility of soluble fibre (TDF-aNDFom-cp) faecal was not affect by the treatments (Tabla 15).

Dietary treatments did not affect (P>0.05) ileal flow of DM, CP and TDF (30.6, 4.70 and 21.7 g/d as average, respectively). The ileal flow of starch was lower in rabbits fed diets with higher soluble fibre content (0.93 vs. 1.32. g/d, respectively; P<0.001) (Table. 16).

### 4.2.3. Mucin production

Ileal mucin concentration varied from 5.06 % of rabbits fed LFS/LThr diet to 6.79% of those fed HFS/HThr and from 0.25 to 0.31% DM in faeces. Ileal and faeces crude protein of mucin was 31 and 25.4% as average, respectably. Ileal and faecal mucin flow increased (P<0.001), with high level of soluble fibre (1.47 vs. 2.13 g/d and 0.060 vs. 0.079 g/d; P=0.033, respectively) (Tabla 17).

### 4.2.4. Mucosa morphology

The increase of soluble fibre level in the diet lead to longer villous height, by 21% (P = 0.001) compared to animals given low soluble fibre (Table 18), whereas, crypt depth was similar between the treatment. As a result, the villous height/crypt depth ratio for rabbits that received low level of soluble fibre was lower compared with animals given high soluble fibre. (4.15 vs. 5.52; respectively P<0.001). The number of goblet cells per villi measured was 15% higher in animal that fed high level of soluble fibre, compared with those that fed low level of soluble fibre (P= 0.0085).

# V. DISCUSSION

The inclusion of sugar beet pulp to increase the dietary soluble fibre in post weaning diets have demonstrated to exert a positive effect on intestinal health of rabbits (Trocino et al., 2013a) probably mediated through changes the intestinal microbiota and in the mucosa integrity, that seems to increase the endogenous substances, and specifically the mucin secretion (El Abed et al., 2011b, 2013). This situation might increase the threonine requirements as observed in rats (Faure et al., 2005).

In our first experiment, there was no mortality in the post weaning period (35-46 d), the growth performance showed relatively high figures, taking into account the sub-limiting faecal digestible lysine content of diets, and there was no effect of soluble fibre inclusion on these traits nor in feed intake, feed efficiency and in the ileal and faecal DM and CP digestibility. However, the inclusion of soluble fibre increased the relative weight of digestive tract and caecum, in spite of the lack of effect on feed intake. These effects are more related with the insoluble fraction than with the soluble fraction of sugar beet pulp (El Abed et al., 2011a). The increase of soluble fibre also decreased caecal pH, suggesting a higher fermentation activity in the caecum as previously described (Gómez-Conde et al., 2009; El Abed et al., 2011a). Our results also confirm a positive effect of the inclusion of soluble fibre on the mucosa integrity, improving the morphology and increased the number of goblet cells in the jejunal mucosa as observed previously (Gómez-Conde et al., 2007; El Abed et al., 2011b, 2013). It might suggest a higher intestinal mucin production (Abad, 2011; El Abed et al., 2011b), although in this experiment it was not measured, which might increase the threonine requirements of the rabbits. However, feeding rabbits under their threonine requirements had no effect on growth performance, mucosa morphology and number of goblet cells, and did not interact with level of soluble fibre. These results are similar than those reported in piglets by Hamard et al. (2007) that did not found any effect of a threonine deficit on growth performance and goblet cells number, although they found a negative effect of a threonine deficit on the villous height/crypth depth ratio. Our results contrast with the negative effect of soluble fibre when pigs were fed a threonine limiting diet on protein deposition (Zhu et al., 2005), although these authors provoked higher differences in soluble fibre among diets than in the present work (0 vs. 12%), or with the positive effect of optimal threonine levels on intestinal mucosal barrier of neonate and weanling

pigs (Law et al., 2007; Wang et al., 2010). It would suggest that threonine requirements just after weaning might be lower than those proposed for the average growing period, which would be in accordance with the results obtained by de Blas et al. (1998). It might be accounted for the relative higher demand of amino acids at weaning for growth than for maintenance, the main destination for threonine, circumstance also observed in pigs (NRC, 2012), even when the type of diet (high in soluble fibre) might have enhanced threonine requirements. On the other hand, this work used recommendations obtained 15 years ago (de Blas et al., 1998) when rabbit growth capacity was lower (around 20%) than that of the rabbits used in this work. These recommendations seems to meet current rabbit requirements which might be explained by a higher efficiency of the utilization of amino acids for growth retention and maintenance in the current hybrids as suggested by García-Palomares et al. (2006).

The second experiment were carried out in a farm with periodic outbreaks of epizootic rabbit enteropathy, and the incidence of mortality in the post weaning period (25-35 d) was significant. The reduction of level of soluble fibre increased the mortality rate confirming its positive effect described by Trocino et al. (2013a). In this study, also the reduction of threonine level under the recommended value tended to impair mortality incidence, which might indicate its limiting content in low threonine diets, in contrast with the results of the first experiment especially with poor sanitary conditions. However, the mortality rate was not recorded in the whole fattening period and we must be carefully in the interpretation of these results. The better health status in rabbits fed high soluble fibre diets accounts for their higher feed intake and the trend to have a higher growth rate. The increase of soluble fibre enlarged the length of the villous, increased the villous height/crypth depth ratio leading to an improvement of ileal starch digestibility, confirming again the already reported positive effect of soluble fibre on mucosa morphology (Gómez-Conde et al., 2007). Furthermore, soluble fibre inclusion also increased the number of goblet cells per villi, and the ileal and faecal flow of intestinal mucins suggesting that its effect on the intestinal mucus layer might be one of the preventive mechanism exerted by soluble fibre, similar to that observed in rats (Ito et al., 2009). In fact, mucin secretion seems to be accompanied by a pool of antimicrobial substances, mainly secreted by Paneth cells in the crypth, encharged to avoid the mucosa colonization (McGuckin et al., 2011). The low of intestinal mucin in the faeces indicated that they are extensively fermented in the caecum (96 %) and might

be another factor influencing the profile of caecal microbiota. In spite of the strong effect of soluble fibre on goblet cells and mucin production in this experiment, or the positive trend observed for threonine content on mortality, no effect of threonine level was reported on mucosa morphology or mucin production, in contrast with previous results in rats and pigs (Faure et al., 2005; Wang et al., 2007). However, dietary threonine improved ileal starch digestibility in low soluble fibre diets with no effect in high soluble fibre diets. This result might indicate that supplementation of threonine in low soluble fibre diets would enhance mucosa functionality (Gómez-Conde et al., 2007). Level of threonine did not modify ileal CP digestibility but faecal CP digestibility decreased for high threonine diets. It could be explained through a more important presence of endogenous substances in faeces, although faecal mucin content was not affected by threonine, or by a higher presence of microorganisms.

The increase of soluble fibre tended to improve the ileal TDF digestibility which is a similar result than those reported by Abad et al. (2012). It is accounted for the increase of both soluble fibre and insoluble fermentable fibre when sugar beet pulp is used for increasing the level of dietary soluble fibre (Abad, 2011). This effect might be related to the changes observed in ileal microbiota profile when dietary soluble fibre is increased (Gómez-Conde et al., 2007; El Abed et al., 2013), although in this study the TDF flow to the caecum was not modified by soluble fibre in contrast to the flow of mucin or that of starch, that tended to be higher for low soluble fibre diets, especially for that with low threonine diet content (LFS/LThr). Faecal digestibility of TDF and NDF also improved with soluble fibre in agreement with Abad et al. (2013) and Trocino et al. (2013a), with no effect of threonine, as expected. Surprisingly, the diet high in both soluble fibre and threonine showed the lowest faecal starch digestibility. It might be attributed to a higher presence of microorganisms that contain minor but appreciable starch content. Microbial protein accounts for 40% of the total CP content in the hard feces (Carabaño et al., 2000), and α-linked glucose starch glucose) content in bacteria was around 2.4% DM (Merry and McAllan, 1983). In contrast with our first experiment, soluble fibre did not influence relative weight of organs that according to the younger rabbits used were higher in the second experiment. Anyway, caecal pH followed the same trend and tended to be acidified with the increase of soluble fibre which is in agreement with the higher faecal digestibility of TDF and NDF reported.

# VI. CONCLUSIONS

The levels of inclusion of soluble fibre and threonine in the diet did not affect the growth performance rabbits, which, might indicate that the inclusion of soluble fibre did not affect threonine requirements for growth.

The increase of soluble fibre improved intestinal mucosa integrity and mucin secretion leading to a better health status of the rabbits when the sanitary conditions worsened. Mucosa barrier traits were not affected by dietary threonine level suggesting that diets met threonine requirements. However, in poor sanitary conditions a low dietary threonine level impaired rabbit health status suggesting a limiting status of this amino acid, although no clear effects on mucosa integrity were detected. Further, researches on this topic are warranted to clarify these effects.

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## Table 8: Ingredients and chemical composition of experimental diets

| | L-SF/ L-Thr | L-SF/ H-Thr | H-SF/ L-Thr | H-SF /H-Thr |
|---|---|---|---|---|
| **Soluble fibre, g/kg** | **89** | **89** | **119** | **119** |
| **Threonine[1], g/kg** | **5** | **6.4** | **5** | **6.4** |
| **Ingredientes, g/kg** | | | | |
| Wheat | 135.6 | 134.2 | 100 | 98.6 |
| Barley meal | 78.7 | 78.7 | 79.6 | 79.6 |
| Sunflower meal | 79.0 | 79 | 97.2 | 97.2 |
| Alfalfa hay | 295.0 | 295 | 195 | 195 |
| Sugar beet pulp | — | — | 126 | 126 |
| Wheat bran | 300.0 | 300 | 300 | 300 |
| Cereal straw | 72.4 | 72.4 | 70 | 70 |
| Soy bean oil | 18.9 | 18.9 | 10 | 10 |
| Alfalfa hay – Yb | 5.0 | 5.0 | 5.0 | 5.0 |
| L-Lysine-HCl | 2.2 | 2.2 | 2.3 | 2.3 |
| L-Treonine | — | 1.42 | — | 1.42 |
| DL-methionine | 0.5 | 0.5 | 1.4 | 1.4 |
| Sodium chloride | 4.0 | 4.0 | 4.0 | 4.0 |
| Calcium carbonate | 5.7 | 5.7 | 6.5 | 6.5 |
| Vitamin and mineral premix[2] | 3.0 | 3.0 | 3.0 | 3.0 |
| **Analyzed composition, g/kg DM** | | | | |
| Dry matter | 919 | 927 | 918 | 924 |
| Ash | 65.9 | 67.1 | 64.1 | 63.6 |
| Crude protein | 164 | 165 | 161 | 158 |
| Starch | 238 | 240 | 219 | 196 |
| Total dietary fibre | 415 | 403 | 448 | 454 |
| Insoluble fibre | | | | |
| aNDFom | 366 | 354 | 379 | 373 |
| aNDFom-cp | 326 | 313 | 336 | 330 |
| Acid detergent fibre | 173 | 172 | 182 | 177 |
| Acid detergent lignin | 34.5 | 37.3 | 34.2 | 33.5 |
| ivDMi2[3] | 356 | 349 | 380 | 393 |
| ivDMi3 | 298 | 296 | 295 | 301 |
| Soluble fibre | | | | |
| TDF-aFNDmo-cp | 88 | 90.0 | 113 | 125 |
| TDF-ivDMi2 | 58.5 | 53.5 | 68.5 | 60.6 |
| CP-TDF, % | 37.2 | 36.6 | 38.8 | 39.6 |
| CP-NDF, % | 24.5 | 24.8 | 27.5 | 28.0 |
| **Calculated composition[1], g/kg** | | | | |
| Lysine | 7.0 | 7.0 | 7.2 | 7.2 |
| Methionine | 2.8 | 2.8 | 3.7 | 3.7 |
| Threonine | 5.0 | 6.4 | 5.0 | 6.4 |
| Calcium | 8.0 | 8.0 | 8.0 | 8.0 |
| Phosphorus | 5.2 | 5.2 | 5.1 | 5.1 |
| Sodium | 2.0 | 2.0 | 2.1 | 2.1 |
| **Calculated faecal digestible content[4], g/kg** | | | | |
| Lysine | 5.7 | 5.7 | 5.7 | 5.7 |
| Threonine | 3.0 | 4.4 | 3.0 | 4.4 |

[1] Calculated value using Tables (Fedna 2010)

[2] Provided by Trouw Nutrition. Vitamin and mineral premix composition (per kg of diet); mg/kg: MgO: 240 mg; S: 240 mg; Mg as $Mg_2O_3$: 20 mg; ZnO: 60 mg; Cu as $CuSO_45H_2O$: 18 mg; I as KI: 1.10 mg; Co as $2CoCO_3.3Co(OH)_2\ H_2O$: 0.30 mg; Se as $Na_2SeO_3$: 0.05 mg; Fe as $FeCO_3$: 78 mg; vitamin A: 9999.9 UI; vitamin $D_3$: 1080 UI; vitamin E like of acetate dl-alpha-tocopherol: 36 UI; vitamin K: 1 mg; vitamin B1: 2 mg; vitamin B2: 6 mg; vitamin B6: 2 mg; vitamin B12: 10 mg; niacin: 50 mg; calcium pantothenate: 20 mg; folic acid: 5 mg; pantothenic acid: 18.4 mg; Biotin: 60 mg; E771 diclazuril 0.5g/100g (clinacox 0.5%): 1 mg; E320 butylhydroxyanisole (BHA): 0.12 mg; E321 butylhydroxytoluene (BHT): 1.32 mg; E324 ethoxyquin: 0.19 mg.

[3] ivDMi2; ivDMi3: in-vitro indigestibility of dry matter 2 and 3 steps, respectively.

[4] Estimated from García et al. (2005) and Villamide et al. (2013).

**Table 9: Effect of level of soluble fibre and threonine on growth traits in rabbits from 35 until 46 d old rabbits (Exp. 1)**

| | Experimental diets | | | | | *P-value* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **L-SF / L-Thr** | **L-SF / H-Thr** | **H-SF / L-Thr** | **H-SF / H-Thr** | **SEM[1]** | **Soluble fibre** | **Threonine** | **Soluble fibre x Threonine** | **BW 35d** |
| **Soluble fibre, g/kg** | **89** | **89** | **119** | **119** | | | | | |
| **Threonine, g/kg** | **5** | **6.4** | **5** | **6.4** | | | | | |
| ADG, g/d | 55.9 | 55.7 | 58.1 | 55.1 | 1.22 | 0.51 | 0.21 | 0.27 | <.0001 |
| ADFI, g/d | 98.8 | 102 | 106 | 101 | 2.66 | 0.27 | 0.69 | 0.17 | <.0001 |
| Feed efficiency, g/g | 0.544 | 0.546 | 0.564 | 0.571 | 0.025 | 0.12 | 0.75 | 0.86 | 0.32 |

[1]n=10 cages (2 rabbits /cage). ADG: average daily gain. ADFI: average daily feed intake.

**Table 10: Effect of level of soluble fibre and threonine on digestive traits of 46 d old rabbits (Exp. 1)**

| | Experimental diets | | | | | *P-value* | | |
|---|---|---|---|---|---|---|---|---|
| | **L-SF / L-Thr** | **L-SF / H-Thr** | **H-SF / L-Thr** | **H-SF / H-Thr** | **SEM[1]** | **Soluble fibre** | **Threonine** | **Soluble fibre x Treonine** |
| **Soluble fibre, g/kg** | **89** | **89** | **119** | **119** | | | | |
| **Threonine, g/kg** | **5** | **6.4** | **5** | **6.4** | | | | |
| Live weight 46 d[2], g | 1495 | 1501 | 1531 | 1495 | 15.6 | 0.33 | 0.33 | 0.17 |
| *Weight of organs (%LW)* | | | | | | | | |
| Digestive tract | 22.2 | 21.5 | 22.6 | 22.8 | 0.45 | 0.032 | 0.52 | 0.27 |
| Stomach | 6.08 | 5.89 | 6.35 | 6.09 | 0.19 | 0.22 | 0.24 | 0.86 |
| Caecum | 7.66 | 7.15 | 7.94 | 8.55 | 0.31 | 0.009 | 0.87 | 0.075 |
| *Caecal pH* | 5.68 | 5.64 | 5.53 | 5.58 | 0.035 | 0.002 | 0.78 | 0.12 |

[1]n=20; [2]For the statistical analysis of live weight at 46 d was used weaning weight at 35 d as covariate (P<.0001).

**Table 11: Effect of level of soluble fibre and threonine on apparent ileal in rabbits at 46 d old and faecal digestibility in rabbits from 42 to 46 d old (Exp. 1)**

| | Experimental diets | | | | | *P-value* | | |
|---|---|---|---|---|---|---|---|---|
| | L-SF/ L-Thr | L-SF/ H-Thr | H-SF/ L-Thr | H-SF/ H-Thr | SEM[1] | Soluble fibre | Threonine | Soluble fibre x Threonine |
| **Soluble fibre, g/kg** | **89** | **89** | **119** | **119** | | | | |
| **Threonine, g/kg** | **5** | **6.4** | **5** | **6.4** | | | | |
| ADFI 42-46, g/d | 63.8 | 62.1 | 60.9 | 63.0 | 1.2 | 0.40 | 0.84 | 0.12 |
| *Apparent ileal digestibility,%* | | | | | | | | |
| Dry matter | 44.2 | — | 45.4 | — | 2.36 | 0.77 | — | — |
| Crude protein | 45.6 | — | 45.1 | — | 5.90 | 0.77 | — | — |
| *Apparent faecal digestibility,%* | | | | | | | | |
| Dry matter | 63.8 | 62.1 | 60.9 | 63 | 1.2 | 0.42 | 0.83 | 0.12 |
| Crude protein | 74.5 | 75.4 | 74.1 | 74.6 | 1.12 | 0.62 | 0.54 | 0.84 |

[1]n = 12 for apparent faecal digestibility; n = 10 for apparent ileal digestibility. ADFI: average daily feed intake.

**Table 12: Effects of level of soluble fibre and threonine on the morphology of the jejunal mucosa in 46 d old rabbits (Exp. 1)**

| | Experimental Diet | | | | | *P-value* | | |
|---|---|---|---|---|---|---|---|---|
| | L-SF/ L-Thr | L-SF/ H-Thr | H-SF/ L-Thr | H-SF/ H-Thr | SEM[1] | Soluble fibre | Threonine | Soluble fibre x Threonine |
| **Soluble fibre, g/kg** | **89** | **89** | **119** | **119** | | | | |
| **Threonine, g/kg** | **5** | **6.4** | **5** | **6.4** | | | | |
| Villous height, µm | 409 | 401 | 529 | 539 | 25.9 | <.0001 | 0.96 | 0.72 |
| Crypt depth, µm | 115 | 113 | 106 | 109 | 3.13 | 0.061 | 0.91 | 0.49 |
| Villous height/crypt depth | 3.60 | 3.55 | 4.90 | 5.07 | 0.30 | <.0001 | 0.95 | 0.86 |
| Nº Globet cell/villi | 12.3 | 14.3 | 19.2 | 17.4 | 1.20 | <0.001 | 0.92 | 0.13 |

[1]n=10.

**Table 13: Effect of level of soluble fibre and threonine on the growth traits and mortality in rabbits from 25 until 35 d (Exp. 2)**

| | Experimental diet | | | | | *P-value* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | L-SF/ L-Thr | L-SF/ H-Thr | H-SF/ L-Thr | H-SF/ H-Thr | SEM[1] | Soluble fibre | Threonine | Soluble fibre x Threonine | Weight 25 d | Litter |
| **Soluble fibre, g/kg** | **89** | **89** | **119** | **119** | | | | | | |
| **Threonine, g/kg** | **5** | **6.4** | **5** | **6.4** | | | | | | |
| n | 27 | 27 | 30 | 31 | | | | | | |
| ADG, g/d | 35.1 | 36.1 | 37.2 | 38.1 | 1.3 | 0.10 | 0.43 | 0.95 | <0.001 | 0.080 |
| ADFI, g/d | 52.3 | 51.9 | 55.1 | 61.4 | 2.13 | 0.004 | 0.16 | 0.11 | 0.058 | 0.12 |
| Feed efficiency, g/g | 0.674 | 0.689 | 0.686 | 0.637 | 0.018 | 0.25 | 0.34 | 0.070 | 0.041 | 0.96 |
| Mortality,% | 17.1 | 14.3 | 5.71 | 0.00 | — | 0.002 | 0.091 | 0.14 | — | — |
| Morbidity,% | 5.71 | 8.57 | 8.57 | 11.4 | — | 0.54 | 0.54 | 0.93 | — | — |

[1]n= 35 for mortality and morbidity trial. ADG: average daily gain. ADFI: average daily feed intake.

**Table 14: Effect of level of soluble fibre and threonine on digestive traits of 35 d old rabbits (Exp. 2)**

| | Experimental diets | | | | | *P-value* | | |
|---|---|---|---|---|---|---|---|---|
| | L-SF/ L-Thr | L-SF/ H-Thr | H-SF/ L-Thr | H-SF/ H-Thr | SEM[1] | Soluble fibre | Threonine | Soluble fibre x Threonine |
| **Soluble fibre, g/kg** | **89** | **89** | **119** | **1119** | | | | |
| **Threonine, g/kg** | **5** | **6.4** | **5** | **6.4** | | | | |
| Live weight 35 d[2], g | 743 | 759 | 785 | 769 | 19.5 | 0.06 | 0.99 | 0.21 |
| *Weight of organs (%LW)* | | | | | | | | |
| Digestive tract | 27.8 | 28.9 | 28.5 | 27.6 | 0.92 | 0.81 | 0.91 | 0.29 |
| Stomach | 7.47 | 7.45 | 7.53 | 7.59 | 0.29 | 0.75 | 0.94 | 0.88 |
| Caecum | 9.36 | 9.48 | 9.26 | 9.4 | 0.29 | 0.73 | 0.67 | 0.98 |
| *Caecal pH* | 5.41 | 5.43 | 5.34 | 5.33 | 0.05 | 0.10 | 0.91 | 0.77 |

[1]n=20; [2]For the statistical analysis of live weight at 35 d was used weaning weight at 25 d as covariate (P<.0001).

**Table 15: Effect of level of soluble fibre and threonine on apparent ileal in rabbits at 35 d old and faecal digestibility in rabbits from 32 to 35 d old (Exp. 2)**

| | Experimental diet | | | | | *P-value* | | |
|---|---|---|---|---|---|---|---|---|
| | L-SF/ L-Thr | L-SF/ H-Thr | H-SF/ L-Thr | H-SF/ H-Thr | SEM[1] | Soluble fibre | Threonine | Soluble fibre x Threonine |
| **Soluble fibre, g/kg** | **89** | **89** | **119** | **119** | | | | |
| **Threonine, g/kg** | **5** | **6.4** | **5** | **6.4** | | | | |
| ADFI 32-35 d, g/d | 52.9 | 52.6 | 56.7 | 60.8 | 2.12 | 0.008 | 0.39 | 0.31 |
| *Apparent ileal digestibility,%* | | | | | | | | |
| Dry matter | 45.6 | 45.9 | 44.5 | 44.9 | 3.11 | 0.73 | 0.89 | 0.99 |
| Crude protein | 50.0 | 49.3 | 46.4 | 47.1 | 4.75 | 0.53 | 0.99 | 0.88 |
| Starch | 89.1[c] | 91.2[b] | 92.6[a] | 92.2[ab] | 0.51 | <0.001 | 0.013 | <0.001 |
| TDF | 5.14 | 5.22 | 13.7 | 15.7 | 5.01 | 0.065 | 0.84 | 0.85 |
| *Apparent faecal digestibility,%* | | | | | | | | |
| Dry matter | 66.4 | 66.0 | 66.3 | 65.5 | 1.10 | 0.65 | 0.37 | 0.81 |
| Crude protein | 81.8 | 80.9 | 81.7 | 79.6 | 0.62 | 0.27 | 0.021 | 0.34 |
| Starch | 98.9[a] | 98.9[a] | 98.4[ab] | 98.3[b] | 0.027 | <0.001 | 0.001 | 0.021 |
| TDF[2] | 33.2 | 31.2 | 37.7 | 39.9 | 1.78 | 0.003 | 0.79 | 0.34 |
| aNDFom-cp | 24.3 | 22.1 | 27.4 | 26.5 | 1.62 | 0.035 | 0.36 | 0.68 |
| [3]Soluble fibre | 69.7 | 70.8 | 74.2 | 76.3 | 3.48 | 0.18 | 0.64 | 0.88 |

[1]n = 11 for apparent faecal digestibility and n = 8 for apparent ileal digestibility. ADFI: average daily feed intake. [a,b,c] Mean values in the same row with a different superscript differ, P <0.05.

[2] TDF: correted by mucin

[3] SF: calculated by the difference (TDF-aNDFom-cp)

**Table 16: Effect of level of soluble fibre and threonine on ileal flow in 35 d old rabbits (Exp. 2)**

| | Experimental diet | | | | *P-value* | | | |
|---|---|---|---|---|---|---|---|---|
| | L-SF/ L-Thr | L-SF/ H-Thr | H-SF/ L-Thr | H-SF/ H-Thr | SEM[1] | Soluble fibre | Threonine | Soluble fibre x Threonine |
| **Soluble fibre, g/kg** | 89 | 89 | 119 | 119 | | | | |
| **Threonine, g/kg** | 5 | 6.4 | 5 | 6.4 | | | | |
| Ileal flow, g/d | | | | | | | | |
| Dry matter | 28.9 | 28.6 | 31.3 | 33.8 | 2.11 | 0.11 | 0.65 | 0.54 |
| Crude protein | 4.43 | 4.40 | 4.85 | 5.10 | 0.42 | 0.21 | 0.82 | 0.74 |
| Starch | 1.38 | 1.12 | 0.91 | 0.93 | 0.12 | <0.001 | 0.16 | 0.083 |
| TDF | 20.9 | 20.3 | 21.8 | 23.9 | 1.65 | 0.18 | 0.64 | 0.41 |

[1]n = 8 for dry matter and protein ileal flow; n=1 for starch and TDF (total dietary fibre) ileal flow.

**Table 17: Effect of level of soluble fibre and threonine on mucin concentration in ileal digesta and faeces in 35 d old rabbits (Exp. 2)**

| | Experimental diet | | | | *P-value* | | | |
|---|---|---|---|---|---|---|---|---|
| | L-SF/ L-Thr | L-SF/ H-Thr | H-SF/ L-Thr | H-SF/ H-Thr | SEM[1] | Soluble fibre | Threonine | Soluble fibre x Threonine |
| **Soluble fibre, g/kg** | 89 | 89 | 119 | 119 | | | | |
| **Threonine, g/kg** | 5 | 6.4 | 5 | 6.4 | | | | |
| *Crude mucin, %* | | | | | | | | |
| Ileal digesta | 5.06 | 5.12 | 6.38 | 6.79 | — | — | — | — |
| Faeces | 0.25 | 0.27 | 0.31 | 0.29 | 0.024 | 0.15 | 0.95 | 0.48 |
| *Crude protein of mucin, %* | | | | | | | | |
| Ileal digesta | 31.7 | 32.4 | 31.5 | 28.5 | — | — | — | — |
| Faeces | 24.7 | 25.0 | 25.5 | 26.5 | — | — | — | — |
| *Mucin flow, g/d* | | | | | | | | |
| Ileum | 1.47 | 1.46 | 2.00 | 2.25 | 0.143 | <0.001 | 0.39 | 0.38 |
| Faeces | 0.056 | 0.063 | 0.083 | 0.075 | 0.008 | 0.033 | 0.95 | 0.41 |

[1]n= 1 for ileal crude mucin concentration; n = 10 for faecal crude mucin %; n= 8 for ileal flow of mucin and n= 10 for faecal flow of mucin; n=1 for crude protein% of ileal and faeces mucin.

**Table 18: Effects of level of soluble fibre and threonine on the morphology of the jejunal mucosa in 35 d old rabbits (Exp. 2)**

| | Experimental Diet | | | | | *P-value* | | |
|---|---|---|---|---|---|---|---|---|
| | L-SF/ L-Thr | L-SF/ H-Thr | H-SF/ L-Thr | H-SF/ H-Thr | SEM[1] | Soluble fibre | Threonine | Soluble fibre x Threonine |
| **Soluble fibre, g/kg** | **89** | **89** | **119** | **119** | | | | |
| **Threonine, g/kg** | **5** | **6.4** | **5** | **6.4** | | | | |
| Villous height, µm | 422 | 417 | 558 | 548 | 26.2 | <.0001 | 0.78 | 0.92 |
| Crypt depth, µm | 102 | 101 | 102 | 97 | 2.80 | 0.59 | 0.34 | 0.43 |
| Villous height/crypt depth | 4.10 | 4.19 | 5.43 | 5.60 | 0.18 | <.0001 | 0.47 | 0.83 |
| Nº Goblet cell/villi | 12.6 | 13.8 | 15.7 | 14.6 | 0.70 | 0.0085 | 0.96 | 0.11 |

[1]n=10.