UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA,

ALIMENTARIA Y DE BIOSISTEMAS

DEPARTAMENTO DE PRODUCCIÓN AGRARIA

**EFFECT OF CELLOBIOSE AND DIETARY
SOLUBLE FIBRE ON FERMENTATION,
GROWTH PERFORMANCE AND HEALTH IN
RABBITS**

TESIS DOCTORAL

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Agrónomo

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Ph.D. THESIS

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PERFORMANCE AND HEALTH IN RABBITS**

By

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DISCLAIMER

Some results of this PhD Thesis are already published:

- Experiment 5: Ocasio-Vega, C., R. Abad-Guamán, R. Delgado, R. Carabaño, M.D. Carro and García, J. *In vitro* caecal fermentation of carbohydrate-rich feedstuffs in rabbits as affected by substrate pre-digestion and donors' diet. World Rabbit Science. In press.

The other results have been submitted for publication in:

- Experiment 1: Ocasio-Vega, C., Delgado, R., Abad-Guamán, R., Carabaño, R., Carro, M.D., Menoyo, D., and García, J. The effect of cellobiose on the health status of growing rabbits depends on the dietary level of soluble fiber. Journal of Animal Science. Submitted.

- Experiments 2, 3 and 4: Ocasio-Vega, C., Delgado, R., Abad-Guamán, R., Carabaño, R., Carro, M.D., and García, J. Effect of cellobiose supplementation on growth performance and health in rabbits. Livestock Science. Submitted.

- Experiment 6: Ocasio-Vega, C., R. Abad-Guamán, R. Delgado, R. Carabaño, M.D. Carro and García, J. Effect of cellobiose supplementation and dietary soluble fibre content on *in vitro* caecal fermentation of carbohydrate-rich substrates in rabbits. Archives of Animal Nutrition. Submitted.

ABBREVIATIONS / ABREVIATURAS

Abbreviation / abreviatura	English	Castellano
ADF	Acid detergent fibre	Fibra ácido detergente
ADFI	Average daily feed intake	Consumo medio diario
ADG	Average daily gain	Ganancia media diaria
ADL	Acid detergent lignin	Lignina ácido detergente
BW	Body weight	Peso corporal
cDNA	Complementary DNA	ADN complementario
CEL	Cellobiose	Celobiosa
CP	Crude protein	Proteína cruda
Ct	Threshold	Valor del ciclo umbral
DF	Dietary fibre	Fibra dietética
DM	Dry matter	Materia seca
ERE	Epizootic rabbit enteropathy	Enteropatía epizootica del conejo
Exp.	Experiment	Experimento
GLU	Glucose (D-+)-glucose)	Glucosa (D-+)-glucosa)
HSF	High soluble fibre diet	Dieta alta fibra soluble
IL	Interleukin	Interleuquina
iNOS	Inducible nitric oxide synthase	Óxido nítrico sintasa inducible
k	Fractional rate of gas production	Rango fraccional de producción de gas
<i>Lag</i>	Initial delay in the onset of gas production	Retraso inicial en el inicio de la producción de gas
LSF	Low soluble fibre diet	Dieta baja fibra soluble
mRNA	Messenger RNA	ARN mensajero
μ_m	Maximum gas production	Rango máximo de

	rate	producción de gas
MUC	Mucin linked membranes	Membranas ligadas a mucina
NDF	Neutral detergent fibre	Fibre neutro detergente
NDOs	Non-digestible oligosaccharides	Oligosacáridos no digestibles
OM	Organic matter	Materia orgánica
<i>P</i>	Probability	Probanbilidad
PCR	Polymerase chain reaction	Reacción en cadena de la polimerasa
PEC	Sugar beet pulp pectins	Pectinas de pulpa de remolacha
rRNA	Ribosomal RNA	ARN ribosomal
SBP	Sugar beet pulp	Pulpa de remolacha
SDF	Soluble dietary fibre	Fibra dietética soluble
SEM	Standard error of the mean	Error estándar de la media
TDF	Total dietary fibre	Fibra dietética total
Ti	Time when μ_m is reached	Tiempo en el que μ_m es alcanzada
TLRs	Toll like receptors	Receptores tipo toll
TNF- α	Tumor necrosis factor alpha	Factor de necrosis tumoral alpha
Vf	Final asymptotic gas production	Asíntota final de producción de gas
VFA	Volatile fatty acids	Ácidos grasos volátiles
WS	Wheat straw	Paja de trigo

SUMMARY

The present thesis constitutes a step forward in advancing the knowledge of the supplementation of cellobiose in the drinking water of rabbits and its possible relationship with soluble fibre level in the diet. There is a positive effect of soluble fibre on rabbit digestive health and therefore on the reduction of mortality in weaning rabbits. Nevertheless, this does not seem to end the problem faced by producers. This thesis aims: 1) to determine the adequate dose of cellobiose and its potential interaction with the dietary soluble fibre level, 2) to characterize the ileal and caecal fermentative profile of the cellobiose when combined with different levels of soluble fibre and 3) to evaluate the influence of cellobiose and soluble fibre supplementation on the *in vitro* caecal fermentation of cellobiose and other carbohydrate-rich feedstuffs. These objectives were developed in six experiments.

In experiment one, the aim of the study was to examine whether the combination of dietary soluble fibre and cellobiose exerts a synergistic effect on growth performance, health status, fermentation traits and immune response in rabbits. Six treatments in a 3×2 factorial arrangement were used: 3 cellobiose concentrations in drinking water (0.0, 7.5 and 15.0 g/L) $\times$ 2 dietary levels of soluble fibre (84.0 and 130 g/kg DM, for the low soluble fibre (LSF) and high soluble fibre (HSF) diets, respectively). A total of 264 young rabbits (35/treatment) were weaned at 34 d of age and had *ad libitum* access to feed and water. At 46 d of age the other 9 rabbits/treatment were slaughtered and ileal and caecal digesta collected to analyze VFA profile and the immune response in the appendix mucosa. At 48 d of age the cellobiose supplementation was withdrawn and the experimental diets were replaced by a standard commercial diet until 61 d of age. From 34 to 48 d of age there was a linear increase of mortality with the level of cellobiose in the HSF group (0 vs. 17.1%; $P = 0.003$). In contrast, a quadratic effect of cellobiose level on mortality was found in the LSF group, the rabbits offered 7.5-cellobiose showing the lowest mortality (5.7 vs. 21.4%; $P = 0.030$). Cellobiose level had a quadratic effect on ADFI, ADG and G:F in this period ($P \leq 0.047$), with the 7.5-cellobiose groups having the best growth performance. In contrast, only minor changes on these traits were observed from 48 d of age onwards. Cellobiose level influenced

quadratically the ileal VFA concentrations ($P = 0.014$), showing the maximal value the 7.5-cellobiose groups. In rabbits fed 7.5-cellobiose-LSF a change of acetate to propionate, butyrate and valerate was found in the ileum ($P \leq 0.070$). Increasing cellobiose levels reduced linearly caecal VFA concentrations in HSF fed rabbits, but no effect was detected in LSF groups ($P = 0.033$). The level of soluble fibre increased VFA concentrations in both the ileum (by 22%; $P < 0.001$) and the caecum (by 11%; $P = 0.005$). The relative gene expression of IL-6, IL-10, and TNF- α , iNOS, MUC-1 and toll like receptors (TLR-2 and TLR-4) in the appendix increased linear and quadratically with increasing levels of cellobiose ($P \leq 0.063$). In conclusion, in rabbits fed LSF diets a dose of 7.5 g cellobiose/L drinking water would be recommended, whereas levels of cellobiose supplementation beyond 7.5 g/L should be avoided in rabbits fed HSF diets.

The second, third and fourth experiments were performed to evaluate the effect of supplement cellobiose (≤ 7.0 g/L) on performance of growing rabbits. In experiment 2, six treatments in a 3×2 factorial arrangement were used: 3 cellobiose concentrations in drinking water (0.0, 3.5, and 7.0 g/L) $\times$ 2 feeds differing in the soluble fibre level (71.9 vs. 130 g/kg DM, for (LSF) and (HSF) diets, respectively). A total of 192 young rabbits (32/treatment) were weaned at 26 d of age and had *ad libitum* access to feed and water. For experiment 3, the same cellobiose concentrations from experiment 2 were evaluated in rabbits fed with a LSF diet (71.9 g/kg DM) and for this propose, 186 young rabbits (62/treatment) weaned at 26 d of age were used. In experiment 4, four treatments in a 2×2 factorial arrangement were structured: 2 cellobiose concentrations in drinking water during the pre-weaning period $\times$ 2 cellobiose concentrations in drinking water during the post-weaning period (0.0 and 7.0 g/L in both periods). For the pre-weaning period, 20 litters (10 kits/litter) were used. Each litter was divided at 22 d of age in two groups (5 kits/group) until 29 d of age. From each group 4 kits/litter were weaned at 29 d of age and assigned at random 0.0 or 7.0 g of cellobiose/L. Cellobiose supplementation affected feed intake and feed efficiency in all experiments ($P \leq 0.045$). In experiment 2, supplemented 3.50 g of cellobiose/L increased ($P = 0.017$) weight gain compared with the others cellobiose concentrations (0.0 and 7.0 g of cellobiose/L), resulting in greater weight at 56 d of age ($P = 0.017$). Results from 26 to 40 d of age in experiment 3 differed from experiment 2, as the increment of cellobiose concentration tended to

decrease feed efficiency ($P = 0.098$). Despite this, from 40 to 50 d of age (experiment 3) this increment the cellobiose concentration increase the feed efficiency ($P = 0.009$). In experiment 4, from 22 to 29 d of age (pre-weaning period), cellobiose supplementation decreased ($P = 0.042$) the feed intake. After weaning, from 29 to 43 d rabbits supplemented with cellobiose, increased ($P \leq 0.001$) the weight gain and the feed efficiency (51.7 vs. 42.4 g/d and 0.457 vs 0.385 g/g, respectively). The increment of cellobiose concentration tended to reduce the mortality in rabbits fed with LSF diets (experiments 2 and 3; $P \leq 0.098$). Contrary, in experiment 4 supplemented the animals with cellobiose in both periods tended to increment the mortality ($P = 0.087$). In conclusion, supplement moderates concentrations of cellobiose (≤ 7.0 g/L) would be recommended for rabbits fed with LSF diets.

In experiment 5, the influence of substrate pre-digestion and donors' diet on *in vitro* caecal fermentation of different substrates in rabbits was investigated. Eight hybrid rabbits were fed two experimental diets containing either low (LSF; 84.0 g/kg DM) or high soluble fibre (HSF; 130 g/kg DM) levels. *In vitro* incubations were conducted using batch cultures with soft faeces as inoculum and four fibrous or fibre-derived, low-starch and low-protein substrates: D-cellobiose (CEL), sugar beet pectin (PEC), sugar beet pulp (SBP) and wheat straw (WS). Substrates in half of the cultures were subjected to a 2-step pepsin/pancreatin *in vitro* digestion without filtration, and the whole residue (soluble, insoluble and added enzymes) was incubated at 40°C. Gas production was measured until 144 h, and volatile fatty acid (VFA) production at 24 h incubation was determined. Cultures without substrate (blanks) were included to correct gas production values for gas released from endogenous substrates and added enzymes. Pre-digestion had no influence on *in vitro* gas production kinetic of WS, and only reduced the time before gas production begins (lag time; by 31%; $P = 0.042$) for SBP, but for both substrates the pre-digestion decreased the molar proportion of acetate (by 9%; $P \leq 0.003$) and increased those of propionate and butyrate ($P \leq 0.014$). For CEL, the pre-digestion increased the gas and total VFA production (by 30 and 114%), shortened the lag time (by 32%), and only when it was combined with LSF inoculum 38 percentage units of acetate were replaced by butyrate ($P \leq 0.039$). Treatments had a minor influence on *in vitro* fermentation traits of SBP. The results showed that the pre-digestion process

influenced the *in vitro* caecal fermentation in rabbits, but the effects were influenced by donors' diet and the incubated substrate. A pre-digestion of substrate is recommended before conducting *in vitro* caecal fermentations. The level of soluble fibre in the donors' diet also influenced the *in vitro* caecal fermentation but its effect depended on the type of substrate.

In the last experiment, the *in vitro* caecal fermentation of five substrates low in starch and protein content [D-(+)-glucose (GLU), D-cellobiose (CEL), sugar beet pectin (PEC), sugar beet pulp (SBP) and wheat straw (WS)] was investigated using as inoculum soft faeces from rabbits receiving different levels of cellobiose and soluble fibre. Twenty four rabbits were supplemented three levels of cellobiose in the drinking water (0.0, 7.5 and 15.0 g/L) and fed two experimental diets containing either low (LSF; 84.0 g/kg DM) or high soluble fibre (HSF; 130 g/kg DM) levels. All substrates were subjected to a two-step pepsin/pancreatin *in vitro* pre-digestion, and the whole residue was used as substrate for the *in vitro* incubations. Gas production was measured until 144 h, and volatile fatty acid (VFA) production was determined at 24 h incubation. Experimental treatments did not affect SBP fermentation and had only a subtle influence on fermentation of WS and GLU. In contrast, fermentation of CEL was markedly affected, and cellobiose supplementation $\times$ donors' diet interactions were detected for most gas production parameters. Both, the fractional gas production (k) and maximal gas production rates were linearly increased ($P \leq 0.025$) and the initial delay in the onset of gas production (*Lag*) linearly decreased ($P < 0.001$) by cellobiose supplementation with the HSF inoculum, with no differences ($P > 0.05$) between the 7.5 and 15 doses. In contrast, with the LSF inoculum cellobiose supplementation only affected k values, which were quadratically increased ($P = 0.043$) and had maximal values for the 7.5 dose. A quadratic effect ($P \leq 0.018$) of cellobiose supplementation was observed for total VFA production at 24 h when CEL and PEC were fermented, obtaining the maximal VFA production for the 7.5 dose of cellobiose. Total VFA production for CEL was greater with LSF than with HSF inoculum (20.7 vs. 12.9 mmol/L; $P = 0.014$), but the opposite was found for WS (3.97 vs. 6.21 mmol/L; $P = 0.005$). The use of LSF-inoculum for CEL fermentation sharply reduced acetate ($P = 0.001$) and increased butyrate proportions ($P \leq 0.001$) compared with the HSF-inoculum. A positive

relationship between total VFA caecal concentrations in rabbits receiving the same experimental treatments and *in vitro* values was only observed when WS was used as substrate ($r = 0.90$; $P = 0.015$; $n = 6$). The results suggest that experimental factors influenced the fermentative activity of caecal digesta, but the observed response differed with the incubated substrate, being the CEL the most affected.

From the results found in this PhD Thesis it can be concluded that a doses of 7.0-7.5 g of cellobiose/L decreased the mortality when the animals where fed with diets low in soluble fibre content. This positive effect might be related with the increased in the butyrate proportion observed *in vivo* and *in vitro*. In contrast, a concentration higher than 7.0 g of cellobiose/L, increased the mortality independently of the soluble fibre content. Accordingly, it would be interesting to evaluate the synergic potential of the cellobiose with other beneficial oligosaccharides (e.g., xylo-oligosaccharides) in order to reduce mortality below the threshold of 10%.

RESUMEN

La presente tesis constituye un avance en el conocimiento de la suplementación de la celobiosa en el agua de bebida de los conejos y su posible relación con el nivel de fibra soluble en el pienso. Existe un efecto positivo de la fibra soluble en la salud digestiva del conejo y por lo tanto, en la reducción de la mortalidad en los conejos destetados. Sin embargo, esto no parece resolver el problema que enfrentan los productores. Esta tesis pretende: 1) determinar las dosis adecuadas de celobiosa para suplementar al conejo y su interacción potencial con el nivel de fibra soluble incluido en el pienso, 2) caracterizar el perfil fermentativo ileal y cecal de la celobiosa cuando se combina con diferentes niveles de fibra soluble, y 3) evaluar la influencia de la suplementación de celobiosa y fibra soluble en la fermentación cecal *in vitro* de celobiosa y otros alimentos ricos en carbohidratos. Estos objetivos fueron desarrollados en seis experimentos.

En el experimento 1, el objetivo fue examinar si la combinación de fibra soluble y celobiosa ejerce un efecto sinérgico sobre los rendimientos productivos, el estado de salud, las características de la fermentación intestinal y la respuesta inmune en conejos. Se utilizaron seis tratamientos estructurados factorialmente (3×2): 3 concentraciones de celobiosa en agua de bebida (0,0, 7,5 y 15,0 g/L) $\times$ 2 niveles dietéticos de fibra soluble (84,0 y 130 g/kg MS, para la dieta baja fibra soluble (BFS) y para la dieta alta fibra soluble (AFS), respectivamente). Un total de 264 conejos (35/tratamiento) fueron destetados a los 34 d de edad y tuvieron acceso *ad libitum* a los alimentos y el agua. A los 46 d de edad, 9 conejos/tratamiento fueron sacrificados y se recogieron muestras de digesta ileal y cecal para analizar el perfil de AGV y la respuesta inmune en la mucosa del apéndice. A los 48 d de edad, se retiró la suplementación con celobiosa y los piensos experimentales se reemplazaron por una dieta comercial estándar hasta los 61 d de edad. De 34 a 48 d de edad hubo un aumento lineal de la mortalidad con el nivel de celobiosa en el grupo de AFS (0 frente a 17,1%, $P = 0,003$). Por el contrario, se encontró un efecto cuadrático del nivel de celobiosa sobre la mortalidad en el grupo BFS, donde los conejos a los que se les ofreció 7,5-celobiosa mostraron la mortalidad más baja (5,7 vs. 21,4%, $P = 0,030$). El nivel de celobiosa tuvo un efecto cuadrático sobre el consumo medio diario

(CMD), ganancia media diaria (GMD) y eficacia alimenticia (G:C) en este período ($P \leq 0,047$), en los grupos de 7,5-celobiosa mostrando el mejor rendimiento de crecimiento. Por el contrario, a partir de los 48 días de edad solo se observaron cambios menores en estas variables. El nivel de celobiosa influyó cuadráticamente en las concentraciones ileales de AGV ($P = 0,014$), mostrando el valor máximo los grupos de 7,5-celobiosa. En conejos alimentados con 7,5-celobiosa-BFS, se encontró un cambio de acetato a propionato, butirato y valerato en el íleon ($P \leq 0,070$). El aumento de los niveles de celobiosa redujo linealmente las concentraciones de AGV cecales en conejos alimentados con AFS, pero no se detectó ningún efecto en los grupos de BFS ($P = 0,033$). El nivel de fibra soluble aumentó las concentraciones de AGV tanto en el íleon (en un 22%, $P < 0,001$) como en ciego (en un 11%, $P = 0,005$). La expresión génica relativa de IL-6, IL-10 y TNF- α , iNOS, MUC-1 y receptores de tipo toll (TLR-2 y TLR-4) en el apéndice aumentaron de forma lineal y cuadrática con niveles crecientes de celobiosa ($P \leq 0,063$). En conclusión, en conejos alimentados con dietas BFS se recomienda una dosis de 7,5 g de celobiosa/L, mientras que estos niveles de suplementación con celobiosa se deben evitar en conejos alimentados con dietas AFS.

El segundo, tercer y cuarto experimento fue realizado para evaluar el efecto de suplementar celobiosa ($\leq 7,0$ g/L) sobre el rendimiento de conejos en crecimiento. En el experimento 2, se utilizaron seis tratamientos estructurados factorialmente (3×2): 3 concentraciones de celobiosa en agua (0,0, 3,5 y 7,0 g/L) $\times$ 2 piensos que difirieron en el nivel de fibra soluble (71,9 vs. 130 g/kg DM, para las dietas baja en fibra soluble (BFS) y alta (AFS), respectivamente). Se utilizaron 192 gazapos destetados a los 26 d de edad, teniendo acceso *ad libitum* a los alimentos y el agua. Para el experimento 3, se evaluaron las mismas concentraciones de celobiosa del experimento 2 en conejos alimentados con una dieta BFS (71,9 g/kg MS), y 186 gazapos destetados a los 26 d de edad fueron utilizados. En el experimento 4, se emplearon cuatro tratamientos en una disposición factorial 2×2 : 2 concentraciones de celobiosa en agua durante un período previo al destete $\times$ 2 concentraciones de celobiosa en agua durante el período posterior al destete (0,0 y 7,0 g/L en ambos períodos). Para la etapa pre-destete, los gazapos 20 camadas (10 gazapos/camada) fueron divididos en dos grupos (5 gazapos/grupo) hasta los 29 d de edad. A los 29 d de edad para la etapa post-destete 4 gazapos/camada fueron destetados

y asignados al azar a dos tratamientos (0,0 y 7,0 g de celobiosa/L), obteniendo cuatro tratamientos. La suplementación de celobiosa afectó el consumo de alimento y eficacia alimenticia en todos los experimentos ($P \leq 0,045$). En el experimento 2, suplementar 3,5 g de celobiosa / L aumentó ($P = 0,017$) su ganancia de peso en comparación con las otras concentraciones de celobiosa (0,0 y 7,0 g de celobiosa/L), resultando en un mayor peso a los 56 d de edad ($P = 0,017$). Los resultados de 26-40 d de edad del experimento 3, difieren de los del experimento 2, observando que el incremento de la concentración de celobiosa tendió a reducir la eficacia alimenticia ($P = 0,098$). A pesar de esto, de 40 a 50 d de edad (experimento 3) este incremento en la concentración de celobiosa aumentó la eficacia alimenticia ($P = 0,009$). En el experimento 4, de 22 a 29 d de edad (periodo previo al destete), la suplementación con celobiosa disminuyó ($P = 0,042$) la ingesta de alimento. Después del destete, de 29 a 43 d conejos suplementados con celobiosa, aumentaron ($P \leq 0,001$) la ganancia de peso y la eficacia alimenticia (51,7 vs. 42,4 g / d 0,457 vs 0,385 g/g, respectivamente). El incremento de la concentración de celobiosa tendió a reducir la mortalidad en conejos alimentados con dietas BFS (experimentos 2 y 3, $P \leq 0,098$). Por el contrario, en el experimento 4 suplementando los animales con celobiosa en ambos períodos tendieron a incrementar la mortalidad ($P = 0,087$). En conclusión, el suplemento de concentraciones de celobiosa ($\leq 7,0$ g/L) se recomienda para conejos alimentados con dietas BFS.

En el quinto estudio, se investigó la influencia de la pre-digestión del sustrato y el pienso de los donantes en la fermentación cecal *in vitro* de diferentes sustratos en conejos. Ocho conejos fueron alimentados con dos piensos experimentales que contenían un nivel bajo (BFS; 84,0 g/kg MS) o alto de fibra soluble (AFS; 130 g/kg MS). Las incubaciones *in vitro* se llevaron a cabo utilizando heces blandas como inóculo y cuatro sustratos fibrosos o derivados de fibra, con bajo contenido de almidón y proteína: D-celobiosa (CEL), pectinas de remolacha (PEC), pulpa de remolacha (PR) y paja de trigo (Paja). En la mitad de de las incubaciones de cada sustrato se realizó una digestión *in vitro* de 2-pasos pepsina/pancreatina sin filtración, y se incubó el residuo completo (parte soluble, insoluble y enzimas añadidas) a 40°C. La producción de gas se midió hasta las 144 h, y se determinó la producción de ácidos grasos volátiles (AGV) a las 24 h de incubación. Se incluyeron cultivos sin sustrato (blancos) para corregir los

valores de producción de gas con el gas liberado de los sustratos endógenos y las enzimas añadidas. La pre-digestión no influyó en la cinética de producción de gas *in vitro* de la Paja, y solo redujo el tiempo de retraso con el que comenzó la producción de gas (*Lag*, en un 31%, $P = 0,042$) para la PR, pero para ambos sustratos la pre-digestión disminuyó la proporción molar de acetato (en un 9%; $P \leq 0,003$) y aumentó las de propionato y butirato ($P \leq 0,014$). Para la CEL, la pre-digestión aumentó el gas y la producción total de AGV (en 30 y 114%), acortó el tiempo de retraso con el que comenzó la producción de gas (en un 32%) y solo cuando se combinó con el inóculo BFS se reemplazaron 38 unidades porcentuales de acetato por butirato ($P \leq 0,039$). Los tratamientos tuvieron una influencia menor en las características de la fermentación *in vitro* de la PR. Los resultados mostraron que el proceso de pre-digestión influyó en la fermentación cecal *in vitro* en conejos, pero los efectos fueron influenciados por el pienso de los donantes y el sustrato incubado. Sería recomendable una predigestión del sustrato antes de realizar fermentaciones cecales *in vitro*. El nivel de fibra soluble en el pienso de los donantes también influyó en la fermentación cecal *in vitro*, pero su efecto dependió del tipo de sustrato.

En el último experimento, se investigó la fermentación cecal *in vitro* de cinco sustratos con bajo contenido de almidón y proteína [D - (+) - glucosa (GLU), D-celobiosa (CEL), pectinas de remolacha azucarera (PEC), pulpa de remolacha azucarera (PR) y paja de trigo (Paja)] utilizando como inóculo heces blandas de conejos que recibieron diferentes niveles de celobiosa y fibra soluble. Veinticuatro conejos fueron suplementados con tres niveles de celobiosa en el agua (0.0, 7,5 y 15,0 g/L) y alimentados con dos piensos experimentales que contenían un nivel bajo (BFS; 84,0 g/kg MS) o alto de fibra soluble (AFS; 130 g/kg MS). Todos los sustratos se sometieron a una pre-digestión *in vitro* de pepsina / pancreatina en dos etapas, y se utilizó el residuo completo como sustrato para las incubaciones *in vitro*. La producción de gas se midió hasta las 144 h, y la producción de ácidos grasos volátiles (AGV) se determinó a las 24 h de incubación. Los tratamientos experimentales no afectaron la fermentación de PR y solo tuvieron una ligera influencia en la fermentación de la Paja y la GLU. Por el contrario, la fermentación de la CEL se vio notablemente afectada, y se detectaron interacciones entre la suplementación de celobiosa y la dieta de los donantes para la

mayoría de los parámetros de producción de gas. Tanto la tasa fraccional de producción de gas (k) como las tasas máximas de producción de gas aumentaron linealmente ($P \leq 0,025$) y la demora inicial en el inicio de la producción de gas (Lag) disminuyó linealmente ($P < 0,001$) mediante suplementación de celobiosa con el inóculo AFS, sin mostrar diferencias ($P > 0,05$) entre las dosis de 7,5 y 15. Por el contrario, con el inóculo BFS la suplementación con celobiosa sólo afectó a los valores de k , que aumentaron cuadráticamente ($P = 0,043$) y tuvieron valores máximos para la dosis de 7,5 de celobiosa. Se observó un efecto cuadrático ($P \leq 0,018$) de la suplementación con celobiosa para la producción total de AGV a las 24 h cuando se fermentaron CEL y PEC, obteniéndose la producción máxima de AGV para la dosis de 7,5 de celobiosa. La producción total de AGV para la CEL fue mayor con el inóculo BFS que con el AFS (20,7 vs. 12,9 mmol/L; $P = 0,014$), pero se observó lo contrario para la Paja (3,97 vs. 6,21 mmol/L; $P = 0,005$). El uso del inóculo BFS para la fermentación de la CEL redujo el acetato ($P = 0,001$) y aumentó las proporciones de butirato ($P \leq 0,001$) en comparación con el inóculo AFS. Se observó una relación positiva entre las concentraciones totales de AGV en conejos que recibieron los mismos tratamientos experimentales y los valores *in vitro* cuando se utilizó la Paja como sustrato ($r = 0,90$; $P = 0,015$; $n = 6$). Los resultados sugieren que los factores experimentales influyeron en la actividad fermentativa de la digestión cecal, pero la respuesta observada difirió con el sustrato incubado, siendo la CEL la más afectada.

De los resultados encontrados en esta tesis doctoral se puede concluir que las dosis de 7,0 y 7,5 g de celobiosa/L disminuyeron la mortalidad cuando los animales fueron alimentados con piensos con bajo en contenido de fibra soluble. Estos efectos positivos podrían estar relacionados con el aumento en la proporción de butirato observado *in vivo* e *in vitro*. Por el contrario, las concentraciones superiores a 7,0 g de celobiosa/L, incrementaron la mortalidad independientemente del contenido de fibra soluble en el pienso. Debido a esto, sería interesante evaluar el potencial sinérgico de la celobiosa con otros oligosacáridos beneficiosos (por ejemplo, xilo-oligosacáridos) para reducir la mortalidad por debajo del umbral del 10%.

I. INTRODUCTION AND OBJECTIVES

The European Union banned the use of antibiotics in 2006 as growth promoters, and their use will be progressively limited removing the main and most efficient tool to control digestive disorders in rabbit farming. This has led the producers, nutritionists and scientists not only to meet the required dietary nutrient profile to optimize rabbit performance, but also to investigate the possible interactions among ingredients/nutrients, gut barrier function and pathogens in order to optimize rabbit health. For this complimentary goal, it is necessary to increase the present knowledge about the supplementation of potential substrates (prebiotics) and their possible synergic effect with the current dietary strategies such the inclusion of a moderate amount of soluble fibre in the diet of growing rabbits (12-14% as fed; Trocino et al., 2013).

Epizootic rabbit enteropathy (ERE) is currently the main digestive disease in rabbit, representing a 71% of the total rabbit diseases. This digestive pathology is responsible for 60% of rabbit mortality in the fattening period with important reductions in feed efficiency and animal growth and it is one of those diseases responsible for the use of antibiotics in this sector. The higher mortality and morbidity rates turn up during the 2 weeks after weaning (Carabaño et al., 2008) stabilizing afterwards.

During the last years, several studies have shown different nutritional strategies that might limit the ERE incidence by modifying different ingredients/nutrients. These modifications are focused on their effects on the gut barrier function, rabbit performance and the mortality. The proposed strategies are: i) to provide a minimal level of insoluble fibre with some specific characteristics (30-32% NDF, >3.7% ADL, and >21% particles larger than 0.3 mm) (Nicodemus et al., 1999 and 2006; Gutiérrez et al., 2002), ii) the inclusion of 12% soluble fibre (Gómez-Conde et al., 2007 and 2009), iii) the reduction of the level of protein (< 16% CP) and the use of specific protein sources (sunflower/plasma) (Gutiérrez et al., 2002 and 2003; García-Ruiz et al., 2006; Carabaño et al., 2009; García-Palomares et al., 2010) or the inclusion of functional amino acids such as glutamine (Chamorro et al., 2010; Delgado et al., 2017).

However, these strategies do not completely solve the problem and further dietary modification may be required looking for a synergistic effect among the different nutrients combined in the diet. The implementations of functional amino acids (arginine and glutamine), n-6/n-3 ratio, or yeast β -glucans (Maertens et al., 2005; Chamorro et al., 2010; Casado et al., 2013; Tanghe et al., 2014; Guenaoui et al., 2015; Delgado, 2017; and El Abed et al. 2015, unpublished) did not show a consistent effect on the rabbit performance or in the reduction of the mortality. In contrast, the positive effects observed by the moderate inclusion of soluble fibre might be partially related to the fermentation/hydrolysis of the fibre in the small intestine that may be responsible for the effects on the intestinal mucosa integrity and functionality and the possible change of the profile of the intestinal microbiota (Gómez-Conde et al., 2007 and 2009; Abad-Guamán et al., 2015). Although the main fermentation site in the rabbit is the caecum, it has been observed that the enzymes present on the small intestine might play an important role in the fermentation of carbohydrates-rich feedstuffs. In fact, the positive effect on rabbit health of carbohydrase supplementation (including fibrolytic enzymes) might be related to the release of low molecular weight oligosaccharides (Gutiérrez et al., 2002; Cachaldora et al., 2004; Pedersen et al., 2015). In this process, oligosaccharides derived from the fibre fraction might influence the intestinal mucosa and/or the microbiota. Accordingly, it might be interesting to supplement indigestible but fermentable substrates such the cellobiose and explore the potential synergism between soluble fibre and cellobiose supplementation. The cellobiose in non-ruminant species (humans, pigs, rats, mice and/or broilers) is slowly hydrolysed/fermented in the small intestine, showing positive effects on the growth performance, improvement of the intestinal barrier and immune response of the animal (Otsuka et al., 2004; Morita et al., 2008; Nishimura et al., 2010; Song et al., 2013; Jiao et al., 2015; Heinritz et al., 2017), although probably depending on the dose it can also influence negatively the animal health (Fischer and Sutton, 1957; Moinuddin and Lee, 1958).

Consequently, the aim of this PhD Thesis was to investigate the capacity of the cellobiose to improve the growth traits and intestinal health status of growing rabbits and the potential synergy with soluble fibre. To accomplish with this general aim, several specific objectives were set out:

1. To evaluate the effect of the inclusion of cellobiose and the effect of the inclusion level of cellobiose:
  - a. Determining the adequate dose of cellobiose and its potential interaction with the dietary soluble fibre level.
  - b. Characterizing the ileal and caecal fermentative profiles of the cellobiose supplementation when combined with different levels of soluble fibre.
2. To evaluate the influence of cellobiose and soluble fibre supplementation on the *in vitro* caecal fermentation of cellobiose and other carbohydrate-rich feedstuffs. A modification of the *in vitro* fermentation methodology was studied to reach this objective.

II. LITERATURE REVIEW

2.1. Dietary fibre

2.1.1. Definition

The concept of dietary fibre (DF) has evolved along time integrating the knowledge of the chemistry of plant cell walls and its nutritional meaning, while the analytical method capabilities have usually been developed later (DeVries, 2003).

Actually, the European Commission integrates these concepts in the regulation 1169/2011/EU that was applied on 13th of December 2014, defining fibre as ‘carbohydrate polymers with three or more monomeric units (degree of polymerization ≥ 3), which are neither digested nor absorbed in the human small intestine, belonging to the following categories: a) edible carbohydrate polymers naturally occurring in the food as consumed; b) edible carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence; c) edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence’.

In biological terms, DF can be defined as the remnants of plants cell walls or analogous carbohydrates resistant to digestion by endogenous enzymes and absorption in the human small intestine (American Association of Cereal Chemistry in 2000 and the Australia New Zealand Food Authority in 2001). However, these components can be partially or totally fermented in the gut (Trowell, 1974; Méheust et al., 2010). Among these indigestible components for humans and animals list polysaccharides as cellulose, hemicelluloses, and pectin, but also other chemical substances such as gums, waxes, protein linked to the cell wall, lignin, and other components from the cytoplasm such resistant starch, oligosaccharides, and fructans.

In the case of herbivores, DF is defined as the indigestible or slowly digested organic matter of feeds that occupies space in the gastrointestinal tract, mainly insoluble fibre (Mertens, 2003). In this case, this definition excludes the rapidly fermenting and soluble carbohydrates, such as oligosaccharides, fructans, and soluble

fibre. This may have several implications due to the positive effects of fermentable fibre (Hall, 2003).

2.1.2. Composition

According to their location, DF components can be divided into two main groups: plant cell wall cytoplasm and components (Selvendran, 1984).

The plant cell wall is the main source of DF. It consists of a series of polysaccharides that are often associated with proteins and phenolic compounds (Selvendran, 1984). Despite this diversity in plant cell wall morphology, two layers are commonly found in the cell walls: the primary (generally a thin, flexible, and extensible layer formed when the cell is growing) and the secondary cell wall (a thick layer formed inside the primary cell wall once the cell is fully-grown; although it is not found in all the cell types). In some cells, such as those in the xylem, the secondary wall contains lignin, which strengthens and waterproofs the wall. Between adjacent plant cells can be found the middle lamella, a thin layer rich in pectin, that glues them together (Theander and Westerlund, 1993). The cell wall constituents can be divided into three groups: fibrillary polysaccharides, matrix polysaccharides, and encrusting substances (e.g., lignin). The fibrillary polysaccharides mainly consist of cellulose (but it usually contains small amounts of non-glycan polysaccharides and glycoproteins). The matrix of polysaccharides is made of linearly orientated polymers (mostly hemicelluloses and pectic substances) (Selvendran, 1984; Selvendran and Robertson, 1990). The encrusting substances are mainly nitrogen compounds.

Dietary fibre is composed of an insoluble fraction constituted by cellulose, hemicellulose, a minor proportion of the pectic substances, lignin, and by a water-soluble fraction consisting in β -glucans, arabinoxylans, and most of the pectic substances (Van Soest, 1978 and 1994; Van Soest et al., 1991). Dividing DF into soluble and insoluble fibre can provide important information for non-ruminants because their recovery in the faeces and impact on the physiological process of digestion are different and thus can explain the effect of fibre in the intestinal physiology and health (Mertens, 2003; Gómez-Conde et al., 2007; Trocino et al., 2013).

Cellulose, the primary component of the insoluble fibre is a structural polysaccharide that usually constitutes the major part of the cell wall. It is considered as a homopolymer, and it is constituted by linear chains of β [1-4] linked *D*-glucopyranosyl units. It is indigestible by the animals due to the lack of enzymes that hydrolyse β linkages (Van Soest, 1994), and can be only fermented by the microbiota.

Hemicelluloses are composed of a group of heteropolysaccharides. It includes xyloglucans, arabinoxylans, β -glucans, arabinogalactans, galactomannans, glucomannans, xylans, and mannans. The hemicelluloses are associated with lignin, which causes that an inverse relation between the digestibility of the hemicellulose and the degree of lignification be found (Selvendran and Robertson, 1990; Van Soest, 1994).

Pectic substances are assigned to the group of acidic plant polysaccharides, with 1,4-linked α -D-galacturonic acid residues (Kertesz, 1951; Van Soest and Wine, 1967). They are considered the most soluble polysaccharides from the cell wall. Pectins are able to embed cellulose and hemicellulose by the formation of a hydrated gel phase. Pectins present heterogeneity in their polysaccharide groups similar to that described for hemicelluloses (Selvendran and Robertson, 1990; Van Soest, 1994). Being exposed to acid pH conditions of < 4.0 or the high concentration of soluble solid, provokes the formation of a gel due to a high degree of esterification in the pectins (such sucrose; Williams et al., 2017).

Lignin is a hydrophobic polymer (non-carbohydrate), formed by a complex three-dimensional network of three phenylpropane units. Lignin serves as a matrix around the polysaccharide components because it provides additional rigidity and strong compression, making the walls hydrophobic and water impermeable (Whetten and Sederoff, 1995). Furthermore, lignin represents an important limitation factor for the animal and the anaerobic digestion system (Van Soest, 1994).

In the plant cell wall, in smaller quantities, other components such minerals (e.g. silica), phenolic acids (linked to hemicelluloses and lignin), some proteins, plant epidermal substances and other phenolic compounds (tannins) can be found (Van Soest, 1994).

The components of cytoplasm, include other indigestible polysaccharides such as resistant starch, fructans, and mannans (DeVries, 2003). Resistant starch is the proportion of starch that is not digested in the small intestine but can be fermented in the large intestine, making it similar to the soluble and fermentable fibre (Haralampu, 2000). Fructans, fructose polymers that are derived from sucrose, possess prebiotic characteristics that result important in nutrition (Ritsema and Smeekens, 2003).

2.1.3. Physiological effects of dietary fibre on performance and health of rabbits

Fibre is the main constituent of rabbit feed, accounting for 40 to 50% of it (Gidenne et al., 2010a). In addition, it is an important energetic substrate for intestinal microbiota. The DF can be affected by physic-chemical properties with nutritional significance such the particle size, hydration properties, cation exchange capacity, viscosity, and absorptive properties of the organic compound (Bach Knudsen, 2001). In the diet, level and type of fibre can affect the rate of passage, the gut flora, the caecal and ileal fermentation, maintenance of intestinal mucosa integrity, and the performance of the rabbits (de Blas et al., 1999; García et al., 2002a; Gidenne et al., 2010a; Trocino et al., 2013). The different types of fibre vary in chemical composition and solubility, which influence their role in the digestive physiology. Dietary fibre has two distinct fractions, insoluble and soluble, and both play an important role in the digestive functions and health of the rabbit.

i. Insoluble fibre

The physiological role of the insoluble fibre (usually measured as neutral detergent fibre (NDF)) has been extensively reviewed in rabbits (Gidenne et al., 2010a,b).

Current recommendations state that diets for rabbits should contain at least 30% NDF to maintain an adequate rate of passage (de Blas and Mateos, 2010). It is also important to take into account the type of insoluble fibre (such the degree of lignification and the particle size) (Nicodemus et al., 1999 and 2006).

The particle size is the major physical property linked to fibre that influences the digestive physiology (García et al., 2000). Fibrous ingredients with a higher proportion of small particles (< 0.3 mm) 1) increase the mean retention time of digesta in the caecum, 2) lead to accumulation of digesta in the caecum increasing its relative weight, 3) enhance fibre fermentation and digestion of fibre, 4) can lead to increased total volatile fatty acids (VFA) concentration and decreased caecal pH, 5) enhances microbial nitrogen recycled through caecotrophy (García et al., 1999 and 2000).

The degree of lignification expressed as dietary acid detergent lignin (ADL) is another important feature of insoluble fibre. A minimal ADL concentration of around 4.1% in growing rabbits has been established in order to ensure the growth performance and health of the animals (Nicodemus et al., 1999). It has been observed that a high or low level of ADL in diet can negatively affect the passage rate, and change caecal fermentation (pH and VFA concentrations) (García et al., 1999, 2000 and 2002b; Gidenne et al., 1999 and 2001)

ii. Soluble fibre

Soluble fibre is the part of DF that comprises the non-starch and non-NDF polysaccharides, including pectic substances, [1-3] [1-4] β -glucans, and fructans (Hall, 2003). In rabbit diets the soluble fibre in spite of its positive effects on health, is not usually measured (Trocino et al., 2013). There are complexities in the methodology to estimate soluble fibre compared with that of insoluble fibre, mainly because most of the methods do not resemble physiological conditions (Monro, 1993). Soluble fibre can be quantify directly (Prosky et al., 1985) or by difference between total dietary fibre (TDF) and NDF (Van Soest et al., 1991). Commonly, soluble fibre is calculated as the difference between TDF and NDF (TDF – NDF), but this value is higher than the value obtained from other methods (Abad et al., 2013). In despite of the method selected to evaluate both, insoluble and soluble fibre a relevant influence is not found on the amount of fermented fibre fractions (Abad-Guamán et al., 2015).

In rabbits, the increment of soluble fibre level in the diet is mostly based on the inclusion of sugar beet pulp. Sugar beet pulp, apart from its insoluble part (e.g.

lignin) possesses pectin substances as major components (Michel et al., 1988). As previously mentioned, the pectin substances are highly soluble and may play an important role in the fermentation along the gastro intestinal tract (mainly at ileal level; Abad-Guamán et al., 2015). The incorporation of moderate levels of dietary soluble fibre (around 12%) as sugar beet pulp in rabbit diets in some studies reduced the feed intake and improved the feed efficiency when the level of soluble fibre increased (Gómez-Conde et al., 2007; Trocino et al., 2010 and 2011). Furthermore, the incorporation of soluble fibre has been shown to improve the morphology, the functionality of the intestinal mucosa and immune response (Gómez-Conde et al. 2007), and to modify the intestinal microbiota (Gómez-Conde et al. 2009). Moreover, it leads to a reduction of the mortality in the post-weaning period in farms affected by ERE (Martínez-Vallespín et al., 2011; Trocino et al. 2013). The soluble fibre may influence indirectly the intestinal mucosa by selecting specific bacteria that modulate immunity. Lindsay et al. (2006) observed that the modulation of cytokines by oligosaccharides in humans is stimulated by bifidobacteria. Meanwhile, in rabbits it has been observed effect of the type and level of fibre on IL-2 production (CD25+ and CD5+CD25) (Gómez-Conde et al. 2007). These positive effects may be related to where the soluble fibre is solubilized or hydrolysed, which may be due to the oligosaccharides that may be released. This can contribute to the changes exerted in the intestinal mucosa, intestinal microbiota and the amount of fermentable fibre both in the small intestine and in the caecum (El Abed et al., 2011 and 2013; Abad-Guamán et. al., 2015).

Weaning rabbits (25-35 days of age) possess a caecal flora that is not as developed as that of the adult animals (Carabaño et al., 2010b; Delgado et al., 2017). According to various authors, this flora is specialized in the fermentation of soluble carbohydrates (Marounek et al., 1995; Gidenne, 1997; Lavrenčič, 2007). The addition of soluble fibre leads to a decrease in the caecal pH and to an increase of the VFA concentration in the caecum (García et al., 2002a). This may be related to a positive effect on the growth reduction of some enteric bacteria (such *Clostridium perfringens*) (Gómez-Conde et al. 2007), but this effect is not yet clear (García et al., 2002a; Gidenne and Licois, 2005).

2.2. NON-DIGESTIBLE DI AND OLIGOSACCHARIDES

As DF, some studies have observed that the addition of oligosaccharides to the diet can positively influence the viability of fattening rabbits.

Oligosaccharides are carbohydrates with a low molecular weight (Mussatto and Mancilha, 2007; de Moura et al., 2015), usually found in small quantities in the common ingredients. They are classified into digestible (mainly derived from starch digestion) or non-digestible by the endogenous enzymes. The non-digestible oligosaccharides (NDOs) are a constituent of dietary fibre (disaccharides are excluded, although some may be considered as DF), and are mainly composed of sugar units as fructose, galactose, glucose, and xylose. The principal NDOs used as nutritional strategies are fructo-oligosaccharides (FOS), α -galacto-oligosaccharides (GOS), xylo-oligosaccharides (XOS), pectin-oligosaccharides (POS), mannan-oligosaccharides (MOS) and cello-oligosaccharides (COS), which show prebiotic properties (de Moura et al., 2015; Falcão-e-Cunha et al., 2007). In fact, the NDOs present physicochemical properties such as higher solubility in water than polysaccharides, taste (depending on the chemical structure, degree of polymerization of the oligosaccharides and the level of mono and disaccharides in the mixture, it can be sweeter than sucrose), viscosity (depending on the molecular weight) and stability (depending on the ring form and the anomeric configuration and linkage type) (Mussatto and Mancilha, 2007). Furthermore, NDOs can resist the digestive process, but despite this, it is possible that some of them can be utilized in the small intestine as shown in pigs (Pedersen et al., 2015). Those that completely or partially resist the digestion on the small intestine reach the caeco-colon (caecum in rabbits). There, by the action of enzymes capable to clave β -linkages and metabolized by anaerobic bacteria through fermentation, NDOs are hydrolysed to small oligomers and monomers (Ziemer and Gibson, 1998). Through this metabolic process, NDOs serve as energy for the bacteria and produce gases (H_2 , CO_2 , CH_4) that are metabolically useless to the host, and small organic acids (short chain fatty acids, (SCFA)), such as acetate, propionate, butyrate, and L-lactate (Mussatto and Mancilha, 2007). This fermentation of the NDOs in the caeco-colon by the bacteria can explain the effects on the animal health. These effects are: 1) modification of the intestinal microbiota; 2) reduction of the pH in the caecum and faeces (the latter not in rabbit); 3) nutrient production (vitamins, B-complex, nicotinic, and folic acid); 4)

increase in the faecal dry weight excretion, but not clear in rabbits; 5) inhibition of diarrhoea; 6) protective effect against infection in the gastrointestinal tract; 7) increase in the absorption of minerals (Mussatto and Mancilha, 2007; de Moura et al., 2015).

The use of NDOs stimulates beneficial microbes found in the gut of the rabbit. With some of the NDOs showing prebiotic characteristics, they have high technological potential and safety advantages (Falcão-e-Cunha et al., 2007). Technological, because NDOs do not have critical problems with the feed processing regarding the thermal conditions and they resist the acidic condition of the stomach. Safety benefits, because they do not introduce foreign microbial species into the gut. Some monosaccharides units derived from the NDOs (such as xylose, mannose, and fructose) can bind to mucosa receptors and thereby help to prevent the adhesion of pathogens to the mucosa, and can furthermore stimulate the gut immune system (Falcão-e-Cunha et al., 2007). Most of the published works are related to human food (Prapulla et al., 2000; Otaka, 2006; Qiang et al., 2009), but positive effects have been also found in farm animals (Zhao et al., 2012; Hajati and Rezaei, 2010). Depending on the NDOs, improvements in the daily gain, feed conversion ratio and/or health status have been observed in pigs and broilers chickens (Patterson and Burkholder, 2003; Lan et al., 2005). In the case of rabbit, the results obtained on the performance vary a lot among studies (Table 1). Furthermore, many studies observed that the use of NDOs as FOS, GOS or MOS increased the VFA and/or had effect on their molar proportions (Peeters J.E. et al., 1992; Morisse et al., 1993; Maertens L. et al., 2004; Mourão et al., 2006; Oso et al., 2013). The mechanisms of action (such as the fermentation) of each NDO depended mostly on their degree of polymerization, sugar and glycosidic linkage and branching, synergy between bacteria during fermentation, relationship between substrate, bacteria and fermentation products (such SCFA), nature of the fermentation and saccharolytic capacity (Mussatto and Mancilha, 2007). Furthermore, a possible interaction may exist with the experimental condition, such as number of animals, hygienic conditions, nature of the NDOs, form of administration of the NDOs (in feed or water), amount of NDOs added to the feed, and the interaction of NDOs used with other ingredients/nutrients present in the feed (Mourão et al., 2006; Falcão-e-Cunha et al., 2007).

Table 1 Effect of oligosaccharides supplementation on performance and mortality of growing rabbits. (Adapted from Falcão-e-Cunha et al., 2007)

Reference	Oligosaccharide ¹	Average daily gain, g/d	Feed conversion, g:g	Mortality (% control vs. % experiment)
Oso et al., 2013	AX	P<0.05 (12.9 vs 13.8)	NS (5.46)	⁻²
Aguilar et al., 1996	FOS	P<0.001 (32.3 vs 35.9)	NS (3.13)	NS (6.1)
Lebas, 1996	FOS	NS (35.5)	NS (3.30)	⁻²
Mourão et al., 2004	FOS	NS (40.4)	P<0.001 (3.6 vs 3.3)	NS (18.1)
Ewuola et al., 2011	FOS	P<0.05 (13.4 vs 14.58)	P<0.05 (7.08 vs 6.53)	⁻²
Gidenne, 1995	GOS	NS (36.2)	NS (2.93)	NS (18.2)
Bónai et al., 2010	Inulin	P<0.05 (47.0 vs 41.5)	NS (1.87)	NS (1.65)
Attia et al., 2015	Inulin	NS (20.1)	NS (5.67)	P<0.01 (17.0 vs 3.70)
Seleem et al., 2007	Lactomannan ^{®3}	P<0.05 (23.7 vs 27.9)	P<0.05 (3.41 vs 2.75)	⁻²
Fonseca et al., 2004	MOS	NS (35.3)	P<0.05 (3.31 vs 2.93)	P<0.01 (11.9 vs 6.3)
Mourão et al., 2006	MOS	NS (39.3)	P<0.05 (3.29 vs 2.92)	NS (5.83)
Bovera et al., 2010	MOS	NS (38.6)	P=0.001 (2.87 vs 2.72)	P<0.05 (34.2 vs 14.2)
Bovera et al., 2011	MOS	P<0.05 (31.0 vs 36.2)	NS (3.48)	NS (11.5)
Oso et al., 2013	MOS	P<0.05 (12.9 vs 19.9)	P<0.05 (5.46 vs 4.22)	⁻²
Attia et al., 2015	MOS	NS (21.1)	NS (5.38)	P<0.01 (17.0 vs 1.60)

¹ AX (arabinoxylans oligosaccharides), FOS (fructo-oligosaccharides), GOS (α -galacto-oligosaccharides) and MOS (mannan-oligosaccharides). ² Mortality % value was under 1% or not included on the study. ³ Lactomannan® (IBEX International Company, Noparia City, El-Behera Province, Egypt), contain mannan, β -glucan, rahmose, xylose as non-starch polysaccharide.

2.2.1. Cellobiose

Cellobiose (CEL) is considered the main component of the group of cello-oligosaccharides, in spite of its degree of polymerization is 2 (chemical structure is show in Figure 1). Cello-oligosaccharides are considered non-digestible carbohydrates, that form part of the functional oligosaccharides group (Satouchi et al., 1996). This group is composed of glucose, binding by β [1-4] linkages produced by chemical and enzymatic hydrolysis from cellulose (Jiao et al., 2015). The cello-oligosaccharides are derived by cellulose from common ingredient used in the animal production feed manufacturing, such wheat straw, rice husk, alfalfa or maize. The CEL is produced from cellulose through the action of specific bacterial enzymes such cellobiosidase and cellulase (Nakamura, 2005). By the action of those enzymes the cellulose is hydrolysed into two molecules of glucose linked by β [1-4] glycosidic bonds similar to cellulose (Kabyemela et al., 1998; Nakamura, 2005).

Figure 1. Chemical structure of cellobiose (Skraup and König, 1901).

Most of the literature about CEL is focused on their use as a biomass substrate for the bio refinery industry (Hasunuma et al., 2013; Parisutham et al., 2017). The CEL can be hydrolysed mechanically or enzymatically. The effects of reaction temperature, pressure and residence time are parameters involved in the mechanic process of decomposition of CEL. This decomposition process induces a hydrolysis and/or a pyrolysis (Bobleter et al., 1986; Park and Park, 2002). Other form to start this hydrolysis process is with the use of sulphuric acid, Bobleter et al. (1986) observed that when CEL was exposed to acid ambient (pH between 2 and 3), the hydrolysis was directly proportional to the acid concentration. Based on the observations previously commented, it can be assumed that CEL could present prebiotic characteristics in animal nutrition.

i. Physiological effects of cellobiose on performance and health

A list of studies where the use of CEL has been evaluated in different ways is shown in Table 2. Satouchi et al. (1996) observed that *in vitro* digestibility of CEL using rat intestinal mucosa as inoculum was 6.3%, and that after oral administration of CEL to rats, the blood glucose concentration showed a gradually increase up to 120 min after administration, implying that CEL was slowly hydrolysed in the lower part of the small intestine. Furthermore, Morita et al. (2008) found that when 60 g of CEL/kg was supplemented in the rats' diet, up to a 64% of added CEL was digested

Table 2. Studies that evaluated the use of cellobiose as a potential additive in the diet.

Reference	Specie	Type of study	Doses used	Observations
Fischer and Sutton, 1957	Rats, calf and pigs	<i>In vivo</i> (laxative action in rats) <i>In vitro</i> (enzymatic hydrolysis with calf, pigs and rats intestine)	0.5 and 0.8g of cellobiose in 5ml of solution 0.1g of cellobiose in 2.0 or 1.0ml of solution.	Cellobiose in rats can have laxative actions in high concentration. Cellobiose is slowly hydrolysed by small intestine mucosa of calf, pigs and rats.
Moinuddin and Lee, 1958	Rats	<i>In vivo</i>	2.2g of cellobiose/kg (15% of the corn-starch add in the diet).	Cellobiose addition result in diarrhoea, decreased in body weight gain and the relative weight of the gut.
Otsuka et al., 2004	Pigs	<i>In vivo</i>	5g of cellobiose/kg	Cellobiose improved the growth performance
Nakamura et al., 2004; Nakamura, 2005	Humans	<i>In vivo</i>	25g of cellobiose in 120ml of warm water.	Cellobiose was hydrolysed/fermented by intestinal microbes.
Fujisawa et al., 2008	Dogs	<i>In vivo</i>	1.4g of cellobiose in combination with <i>Lactobacillus</i>	Cellobiose in combination of <i>Lactobacillus</i> offered protection against faecal disorders.
Morita et al., 2008	Rats	Experiment 1 <i>In vivo</i> digestibility of cellobiose Experiment 2 <i>In vitro</i> digestibility of cellobiose Experiment 3 <i>Caecal fermentation</i> of cellobiose	Experiment 1 60g of cellobiose/kg Experiment 2 0.5 to 30 mmol of cellobiose/L Experiment 3 30 or 60 g of cellobiose/kg	Cellobiose was digested in small intestine by β -galactosidase.
Nishimura et al., 2010	Mice	<i>In vivo</i>	6.0 or 9.0% of cellobiose wt/wt	Cellobiose presents a preventive effect against DSS colitis ¹ .
Yang et al., 2010	Rabbits	<i>In vitro</i>	500 mg/5 ml inoculum (150 g of caecal content + medium solution)	Cellobiose fermentation increased gas production, total VFA and butyrate production.
Hasunuma et al., 2011	Calves	<i>In vivo</i>	5g of cellobiose/day (in drinking milk and post weaning in warm water)	Cellobiose improved the daily body weight gain and the feed efficiency.
Song et al., 2013	Broilers	<i>In vivo</i>	1.5g of cellobiose/kg	Cellobiose improved intestinal microflora, morphology and barrier integrity.
Uyeno et al., 2013	Calves	<i>In vivo</i>	5g of cellobiose/day pre-weaning and 10g/day post weaning.	Cellobiose supplementation modulated the intestinal microbiota.
Jiao et al., 2014 and 2015	Pigs	<i>In vivo</i>	1.5, 3.0 and 4.5g of COS ² (84.2% of cellobiose)/kg	Cellobiose improved the intestinal barrier funtion, intestinal microflora, mucosa architecture and nutrient transport in the small intestine.
Heinritz et al., 2017	Pigs	<i>In vitro</i>	5.0, 10.0, 15.0, 20.0, 25.0, 30.0 and 100 g of cellobiose/kg	Cellobiose concentrations linearly increased the gas production and the total VFA. Cellobiose improved the gut health

¹DSS colitis (Dextran sulphate sodium induced experimental colitis). ²COS (cello-oligosaccharide).

by the enzyme β -galactosidase present in the small intestine. In contrast, Nakamura (2005) in a study with humans observed that the administration of 25 g of CEL/120 ml of warm water did not show any increase in the glucose level in the blood, but it was observed a higher excretion of hydrogen gas after CEL ingestion. This can indicate that in humans, part of the administrated CEL escapes the digestion and might be fermented in the large bowel. Furthermore, the supplementation of CEL in humans contributed approximately 2 kcal/g of available energy (Nakamura, 2004 and 2005). The difference among different trials can be due to the reduction of some enzymes (lactase) as the human grows (Morita et al., 2008). In humans it has been observed that the activity of lactase, an enzyme that cleaves the β [1-4] glycosidic bond, is different according to the age, sex, and race (Bayless, 1966; Rao et al., 1994).

Cellobiose supplementation improved the daily weight gain in weanling pigs (Otsuka et al., 2004) and changed the microbial ecosystem, that possibly contributed to the improvement of the intestinal barrier functions (Jiao et al., 2015). These effects might be related to the increase of the immune response in the animal. In mice (infected with colitis) fed with CEL (9% wt/wt) the mucosal mRNA expression of inflammatory cytokines (such as TNF- α) was decreased (Nishimura et al., 2010). Furthermore, the inclusion of CEL apparently influences the fermentation in the gastro intestinal tract. It has been observed that the increase of CEL concentration (up to 60 g/kg in rats and up to 30 g CEL/kg in pigs) increased the total VFA and modified the proportions of VFA produced (mainly the acetate) (Otsuka et al., 2004; Morita et al., 2008; Heinritz et al., 2017). Besides the effects of the addition of CEL, it will be of interest to evaluate the administrated concentrations and their possible synergic effect with the diet or with the microbiota present in the tract of the animal. Fujisawa et al. (2008) in a study with dogs observed that adding CEL in combination with *Lactobacillus spp.* (as probiotic) positively modified the intestinal microbiota. In contrast, the increased of CEL concentration (up to 0.8 g of CEL infused directly to the stomach and up to 15% of CEL as replacement in high corn-starch diet in rats) increased the laxative action, causing loss of weight (Fischer and Sutton, 1957; Moinuddin and Lee, 1958). In rabbits, studies of the use of CEL are scarce. Feng et al. (2009), looking for characterized biochemical properties of β -glucosidase utilizing content from rabbit caecum, found that this enzyme hydrolysed cello-

oligosaccharides (including cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose) under pH and temperatures similar to the biological condition of the animal (6.5 and 39°C, respectively). Lactase activity in rabbits is very high until weaning, and then, due to the change of milk to solid feed, decreases (Marounek et al., 1995; Gutiérrez et al., 2002; García Rebollar et al., 2004). This means that the prebiotic potential of the cello-oligosaccharides, especially the cellobiose, must be evaluated in rabbits at weaning stage. The positive effects reported for the addition of cellobiose might be complementary with those exerted by soluble fibre in the diet of rabbits.

2.2.2. *In vitro* methods to study intestinal fermentation

The fermentation occurs along the gastro intestinal tract of non-ruminants, producing VFA, gases (CO₂, H₂, and CH₄) and microbial cells (Robinson et al., 1989; Jensen and Jørgensen, 1994). The gas produced is mainly due to the fermentation of carbohydrates (cellulose, hemicellulose, pectins and minor proportion of starch). This anaerobic fermentation produces other compounds (succinate, lactate, carbonate skeletons, etc.) that can be used by the microbiota for their own growth (Opatpatanakit et al., 1994; Schofield et al., 1994). Furthermore, the gas production can also be derived from protein fermentation (Makar, 2004) or from endogenous substances (Marounek et al., 2000; Tran et al., 2016).

In rabbits, the microbiota present along the gastro intestinal tract is involved in the fermentation process, but this population is influenced by the type of diet received (Carabaño et al., 2010b). Up to 40% of the energy requirements of rabbits in maintenance and fed with diets rich in fibre are obtained from VFA produced by the microbiota fermentation in the caecum (Parker, 1976; Marty and Vernay, 1984). Despite this, there may be a fermentation/hydrolysis in the ileum although limited to the soluble and part of the insoluble fibre (El Abed et al., 2011; Abad-Guamán et al., 2015). These fractions may be related to the positive effects of the soluble fibre on the mucosa of the jejunum (Gómez-Conde et al. 2007). However, it is not clear i) whether the relatively high digestibility values of fibre fractions in the ileum reported by Abad-Guamán et al. (2015) are due to fermentation or only to their hydrolysis, and ii) whether the effect on the jejunal mucosa is direct and/or mediated by

microbiota (though VFA or other mechanism). In addition, the quantification of VFA concentration in the digesta is not a good estimation of VFA absorption (Van der Klis and Jansman, 2002). In rabbits, the VFA concentration follows a circadian rhythm as feed intake and faecal excretion (Gidenne, 1986; Bellier et al., 1995). The caecal VFA concentration is greater during the period of hard faeces excretion, whereas that in the ileum is much lower but constant along the day (Fioramonti and Ruckebush, 1976; Gidenne, 1986; Gidenne and Bellier, 1992; Bellier et al. 1995; Bellier and Gidenne, 1996).

In order to enhance the understanding of intestinal fermentation, *in vitro* techniques have been developed focused on the caecal fermentation in rabbits (Bovera et al., 2008 and 2009) or on faecal fermentation in pigs (Bindelle et al., 2007b) derived from those used in ruminants. Menke et al. (1979) developed a rapid method for measuring gas production during incubation of feedstuffs with rumen liquor. *In vitro* gas production methodology is based on the fact that anaerobic digestion of carbohydrates by digestive microorganisms produces gas (CO₂, CH₄ and traces of H₂) and VFA (mainly acetate, propionate and butyrate). Gas production can be measured to estimate the rate and extent of feed degradation (Getachew et al., 1998). This *in vitro* technique require substrates, an anaerobic medium and a representative sample of the microbiota population present in the part of GIT in which fermentations occur (donors' diet). This procedure has been adapted to study the fermentative process of non-ruminant species (pigs, equines, poultry and rabbits), but their utilization is still limited (Fondevila et al., 2002; Bovera et al., 2006 and 2009; Abdouli and Attia, 2007; Song et al., 2013).

Factors such as the type of substrate, processing of the substrate prior to the incubation, animal donors' diet, and the type of inoculum (ileal, caecal and/or faecal content) affect the gas production methodology (Bauer et al., 2003; Awati et al., 2005 and 2006; Azarfar et al., 2007). In rabbits, the use of *in vitro* techniques is limited and not standardized. The differences in the methodology used among studies done with rabbits as donors are shown in Table 3. There is an important variation in the amount of substrate used and the inoculum/substrate ratio employed on the *in vitro* procedures. Abad-Guamán et al. (unpublished) compared in rabbits, how the type of inoculum (ileal, caecal and/or faecal) and the dose used (g fresh digesta/g

Table 3. *In vitro* fermentation methodology implemented in different studies with rabbit inocula.

Reference	Inoculum	g substrate/vial	Substrate pre-treatment	g inoculum/vial	g inoculum/ g substrate	n ²
Piattoni et al. 1997	Caecal	0.50	No	8.30	16.6	6
Calabró et al. 1999	Caecal	0.82	No	2.50	3.05	4
Pascual et al. 2000	Caecal	1.00	Si	4.50	4.50	3
	Faecal	1.00	Si	4.50	4.50	3
Stanco et al. 2003	Caecal	0.82	No	2.50	3.05	4
Bovera et al. 2006, 2008 and 2009	Caecal	1.00	No	3.33	3.33	3-4
	Faecal	1.00	No	1.11	1.11	3-4
Lavrenčič 2007; Kermauner and Lavrencic 2010	Caecal	0.175	Si	0.59	3.37	3-4
El-Adawy et al. 2008	Caecal	0.50	No	0.15	0.30	4
		0.50	No	0.06	0.12	4
Yang et al. 2010	Caecal	0.50	Si	2.50	5.00	8
Belenguer et al. 2011	Caecal	0.80	No	8.18	10.2	4
Rodríguez-Romero et al. 2011	Caecal	0.80	Si	8.00	10.0	6
Abad-Guamán et al. 2018	Ileal	0.20	Si	0.10	0.50	2
		0.20	Si	0.20	1.00	2-3
		0.20	Si	0.30	1.50	3
		0.20	Si	0.40	2.00	2-3
	Caecal	0.20	Si	0.10	0.50	2
		0.20	Si	0.20	1.00	2
		0.20	Si	0.40	2.00	2
		0.20	Si	0.40	2.00	2

¹ Dried. ² Number of replicates.

substrate) influence the *in vitro* fermentation. Comparing different doses (0.5, 1.0, 1.5 and 2.0 g of fresh digesta/g substrate) they observed that 1.0 g fresh digesta/g DM substrate was adequate for both ileal and caecal inocula for *in vitro* incubations with rabbits digesta. Furthermore, both, the ileal and caecal gas production parameters were positively correlated with those obtained with the hard and soft faeces inocula, respectively ($r \geq 0.77$; $P \leq 0.040$). This indicates that soft and/or hard faeces could substitute the caecal inoculum in *in vitro* studies, avoiding the slaughter of rabbits (Abad-Guamán et al., unpublished). In the other hand, it can be observed that in most of the studies (Calabro et al., 1999; Bovera et al., 2006 and 2009; Lavrenčič, 2007; Yang et al., 2010) the substrate was just grounded, but in others studies with pigs and rabbits (Bindelle et al., 2007ab; Rodríguez-Romero et al., 2011 and Abad-Guamán et al., unpublished) the substrate was further subjected to an *in vitro* pre-digestion procedure with pepsin and pancreatin in order to simulate the digestion in the stomach and small intestine, respectively. This enzymatic pre-digestion procedure seems to be more appropriate, in order to exert potential changes in the substrate similar to those produced during the digestion in the stomach and small intestine and to provide some endogenous substances for caecal fermentation (Coles et al., 2005). The use of the pre-digestion generally involves the use of the insoluble residue, obtained by filtration or centrifugation, as substrate for *in vitro* caecal fermentation (Rodríguez-Romero et al., 2011) in rabbits or the evaluation of fermentation in pigs (Bauer et al., 2003). However, this methodology might result questionable due to the loss of the soluble fibre during the filtration/centrifugation process, especially in low protein substrates. In addition, donors' diet has been shown to influence the *in vitro* gas production and substrate degradability in pigs and ruminants (Bindelle et al., 2007ab; Mateos et al., 2013; Sappok et al., 2013), but the information is limited in rabbits (Rodríguez-Romero et al., 2011).

2.2.3. Water intake

The water intake in rabbits has an important correlation with the feed intake. Rabbits fed a pelleted diet have a water requirement that exceeds the dry matter intake (Table 4). Despite this, Prud'hon et al. (1975) observed that animals consumed water almost the same number of times that they consumed feed during the day

(Table 4). This water intake increased with the age and depended on the physiological status (lactating or not).

The limitation of water intake in rabbits has been used to restrict feed intake (Boisot et al., 2004 and 2005; Verdelhan et al., 2004). The ratio of water to feed intake seems to increase with age: from 1.56 to 1.86 in growing rabbits of 6 and 18 wk of age, and reach 2.0-2.1 for breeding rabbits (Gidenne et al., 2010c). In contrast, Delgado et al. (2015) did not find this effect during the fattening period, but observed that this ratio varied with the dietary soluble fibre (Table 5). From 26 to 39 d of age, rabbits fed high soluble fibre diets reduced their feed intake and increased their water intake compared to animals fed a low soluble fibre diet, resulting in a greater water/feed intake ratio (2.14 vs 1.46; Delgado et al. 2015).

Environmental factors such as the temperature can affect the water and feed intake in rabbits. Eberhart (1980) reported that the increase of the temperature decreased the feed intake and increased the water intake (temperatures from 10° to 30°C). Rabbits presenting signs of disease (such ERE) reduced their feed intake faster than the water intake (Delgado et al. 2015). Based on this it can be hypothesized that the supplementation of feed additives in the water can be a good methodology to improve the growth and health of animals. Besides, the inclusion of additives via the drinking water results economically a better option.

Table 4. Feeding and drinking behavior of the domestic rabbit from 6 to 18 weeks of age (n = 9; adapted from Prud'hon et al., 1975. Cited by Gidenne et al., 2010c).

	Age in weeks		
	6	12	18
Feed			
Feed intake, g/d	98	194	160
No. meals/d	39	40	34
Drinking water			
Water intake, g/d	153	320	297
No. drinks/d	31	28.5	36
Water/feed intake	1.56	1.65	1.86

According to the EFSA (2010), the feed additives are intentionally added to feed or water in order to perform one or more functions beneficial to the animal (Regulation (EC) No 1831/2003, Article 2, 2a). Furthermore, the EFSA (2010) indicated that the additives for use in drinking water should be water

soluble/miscible or at least able to form stable suspensions in water with a demonstrated homogeneity of distribution and stability of the active substance at least for 24 h. The CEL solubility in water is around 12-20% (depending of temperature; Nippon Paper Industries Co., Tokyo, Japan; utilized in this PhD Thesis), what means that it may be supplemented in the drinking water of rabbits.

Table 5. Effect of the level of dietary soluble fibre on the water intake of healthy growing rabbits (n = 10; adapted from Delgado et al., 2015).

	Experimental diets			P-value
	Low soluble fibre	High soluble fibre	rsd	Soluble fibre
26-39 d of age				
Water intake, g/d	111	142	37.8	0.055
Feed intake, g/d	72.7	66.9	7.46	0.065
Water/feed intake	1.46	2.14	0.57	0.023
39-62 d of age				
Water intake, g/d	206	214	47.0	0.68
Feed intake, g/d	145	142	12.3	0.56
Water/feed intake	1.43	1.50	0.33	0.58
26-62 d of age				
Water intake, g/d	164	188	35.3	0.16
Feed intake, g/d	120	115	9.51	0.26
Water/feed intake	1.32	1.63	0.23	0.012

In pig production it is common to administer feed additives in the drinking water of the animals (Mavromichalis, 2016). Moreover, during the last years, the implementation of liquid diets has shown positives effects on performance and health of pigs (Brooks et al., 2001). The inclusion of beneficial substances in liquid form, allows the supplementation of the animal with different additives in different doses and time intervals. This gives the producer the freedom to have a better rotation of medication or additives used on a daily basis. To our knowledge, information on studies conducted to compare the effect of the included additive in drinking water or in the diet is scarce. Supplemented cello-oligosaccharides (such as CEL) in the drinking water (Hasunuma et al., 2011) and/or in the feed of calves (Uyeno et al., 2013), exert positive effects although it does not seem to affect the mechanism of action in the animals. However, from these results it cannot be concluded which is the best way to supplement it.

III. MATERIALS AND METHODS

3.1. EXPERIMENTAL DESIGN

All procedures involving animals were carried out in accordance with the Spanish guidelines for experimental animal protection (Spanish Royal Decree 53/2013; BOE, 2013) after being approved by the Animal Ethics Committee of the Universidad Politécnica de Madrid.

3.1.1. Objective 1:

Determine the adequate dose of cellobiose and its potential interaction with the dietary soluble fibre level in rabbits. Characterize the ileal and caecal fermentative profile of the cellobiose when combined with different levels of soluble fibre.

In order to reach this objective four experiments were conducted.

3.1.1.1. Experiment 1: “The effect of cellobiose on the health status of growing rabbits depends on the dietary level of soluble fibre”.

Six treatments in a 3×2 factorial arrangement (3 cellobiose concentrations $\times$ 2 dietary levels of soluble fibre) were used. Three concentrations of cellobiose were used in the drinking water: 0.0, 7.5 and 15.0 g/L (D-cellobiose, NPC Cello-Oligo, Nippon Paper Industries Co., Tokyo, Japan. According to the manufacturer contained 96.6% cellobiose β 1–4, 1.9% cello-oligosaccharide, 1.5% glucose, and no nitrogen). These concentrations were selected to obtain a wider range of cellobiose supplementation than those used in previous studies conducted with poultry and pigs (0.3-0.6% in the diet; Song et al., 2013; Jiao et al., 2014). Cellobiose was supplemented in drinking water because in sick rabbits the water intake is less affected than feed intake that is clearly reduced compared with healthy rabbits (Delgado et al., 2015). Two experimental diets were formulated to differ in their dietary soluble fibre concentration (84.0 and 130 g/kg DM, for the low soluble fibre (LSF) and high soluble fibre (HSF) diets, respectively) and starch (226 and 182 g/kg

DM for LSF and HSF, respectively). The increase of soluble/fermentable fibre was obtained by replacing wheat straw and bran in the LSF diet by sugar beet pulp, that not only provides of soluble fibre but also of insoluble fermentable fibre. Ingredients and chemical composition of diets are shown in Table 1.

A total of 264 crossbred mixed-sex rabbits (New Zealand White $\times$ Californian, V $\times$ R from UPV, Valencia, Spain) weaned at 34 d of age were used in this study.

For the growth trial, 210 rabbits (779 ± 7.3 g BW) were blocked by litter, randomly assigned to each of the six experimental treatments (35 rabbits/treatment) and housed individually with *ad libitum* access to feed and water. The ADFI, ADG and mortality were recorded individually. Water intake could not be individually measured and was estimated according to the water/feed intake ratio previously reported in rabbits fed the same experimental diets by Delgado et al. (2015). Before weaning rabbits had access to their mothers' feed (180 CP, 329 NDF, and 93.4 soluble fibre, all in g/kg DM). At 48 d of age the supplementation of cellobiose in the drinking water was withdrawn and the experimental diets were replaced by a standard commercial diet (164 CP, 341 NDF, and 58.7 soluble fibre, all in g/kg DM) for all rabbits. The experiment finished at 61 d of age.

To evaluate the ileal and caecal fermentation, sucrose activity and immune response, Another 54 rabbits (620 ± 14.7 g BW) were also blocked by litter, and randomly assigned to one of the six treatments (9 rabbits/treatment). They were housed individually and had *ad libitum* access to feed and water. After 12 d adaptation period, they were slaughtered by head concussion between 19:00 and 21:00 h. After slaughter, the whole gastrointestinal tract was removed and weighed. The caecum was removed and its full weight was recorded. The caecal content was then extracted, weighed, homogenized, and the pH immediately measured with a Crison Basic 20 pHmeter (Crison Instruments, Barcelona, Spain). About 2 g of caecal content were weighed, mixed with 2 mL of 0.5 N HCl, and immediately frozen (-20°C) until analysis of VFA concentrations by gas chromatography as described by Carro et al. (1992). The pH of the ileal content was measured and a sample (1 g) was taken for VFA analysis. The remaining caecal and ileal content was used to determine DM content. In addition, 6 cm samples were excised from the middle part of the jejunum, flushed with saline solution, frozen in dry ice, and

Table 1. Ingredient and chemical composition of the experimental diets.
(Experiments 1, 5 and 6)

	Low soluble fibre (LSF)	High soluble fibre (HSF)
Ingredient, g/kg as-fed		
Wheat bran	280	130
Wheat straw	100	50.0
Beet pulp	0.00	180
Sunflower meal 29	99.7	130
Dehydrated alfalfa 13	150	150
Soybean meal 44	80.0	80.0
Wheat grain	227	217
High oleic sunflower oil	8.50	8.50
Sunflower oil	21.5	21.5
L-Lysine HCl	4.40	4.40
DL-Methionine	0.80	0.60
L-Threonine	3.10	3.20
Calcium carbonate	12.0	7.00
Sodium chloride	3.00	3.10
Calcium phosphate	5.00	10.0
Vitamin/mineral premix ¹	5.00	5.00
Analysed chemical composition, g/kg DM		
DM	908	908
Ash	70.8	67.5
CP	167	165
Total dietary fibre (TDF)	391	442
NDF ²	307	312
ADF ³	165	185
ADL ³	31.0	33.0
Soluble fibre	84.0	130
Starch	226	182
Ether extract	53.8	48.7
Sugars	79.9	81.7

¹ Provided by Trouw Nutrition (Madrid, Spain). Mineral and vitamin composition (per kg of complete diet): 20 mg of Mn as MnO; 59.2 mg of Zn as ZnO; 10 mg of Cu as CuSO₄ 5H₂O; 1.25 mg of I as KI; 0.495 mg of Co as CoCO₃ H₂O H₂O; 76 mg of Fe as FeCO₃; 8375 UI of vitamin A; 750 UI of vitamin D₃, 20 UI of vitamin E as DL- α -tocopherol acetate, 1.0 mg of vitamin K; 1.0 mg of vitamin B₁; 2 mg of vitamin B₂; 1 mg of vitamin B₆; 20 mg of Niacin; 54.1 mg of Betaine; 137.5 mg of Choline chloride; 66 mg of robenidine; 50 mg of ethoxyquin. ² Values were corrected for ash and crude protein. ³ Values were corrected for ash.

immediately stored at -20°C to determine sucrose activity as described by Gómez-Conde et al. (2007). Finally, 2 cm-segments of the distal part of the appendix were taken to characterize the immune response in 8 rabbits/treatment. Samples were cleaned with saline solution (NaCl, 0.9%), cut longitudinally and scraped to obtain approximately 50 mg of mucosa. Samples were placed in vials containing 1 ml of RNA preserving solution (RNA Later, Applied Biosystems, Foster City, CA, USA) and frozen at -80°C. Tissue disruption for RNA isolation from appendix was performed using Trizol reagent (Sigma-Aldrich, St Louise, MO, USA) and a mixer mill MM-200 (Restch, Stuttgart, Germany). Total RNA was isolated using the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St Louise, MO, USA) according to manufacturer's instructions. A DNase treatment step using RNase-Free DNase Set (Qiagen, Hilden, Germany) was added to prevent genomic DNA contamination. The RNA concentration was measured by spectrophotometry (Epoch™, BioTek, Winooski, VT, USA) combined with the Take3™ Micro-Volume Plate (BioTek, Santa Barbara, CA, USA). The extracted A_{260}/A_{280} ratio was used to calculate the quantity of diluted RNA for the following reverse transcription.

First strand cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The relative gene expression of selected cytokines was determined using real-time, quantitative PCR. The specific primers for rabbit glyceraldehyde-3 phosphate (GADPH) and hipoxantine-guanine phosphoriltransferase (HPRT) (housekeepings), IL-10 and tumor necrosis factor-alpha (TNF- α) were taken from Godornes et al. (2007) and Chamorro el al. (2010). Primers for TLR-2, TLR-4, MUC-1, and MUC-13 were taken from Bäuerl et al. (2014) and those for IL-6 and the inducible nitric oxide synthase (iNOS) were designed by using Primer Express® v.2 (Applied Biosystems, Foster City, CA, USA). The specificity of the amplified product was confirmed through melting curves analysis and further confirmed by gel electrophoresis. The quantitative PCR was performed in an ABI Prism 7300 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). Each reaction mix consisted in around 100 ng of first strand cDNA as a template, specific primers, ultrapure water and SYBR® Green Master Mix (Applied Biosystems Foster City, CA, USA) as fluorescent DNA intercalating agent. All samples were run in

triplicate and quantified by normalizing the target gene signal to that of the GAPDH and HPRT geometric mean.

3.1.1.2. Experiment 2: “Effect of cellobiose supplementation on growth performance and health in rabbits”.

Six treatments in a 3×2 factorial arrangement were used (3 cellobiose concentrations in drinking water $\times$ 2 dietary levels of soluble fibre). Three concentrations of cellobiose in the drinking water were used (0.0, 3.5 and 7.0 g/L) by diluting D-cellobiose (NPC Cello-Oligo, Nippon Paper Industries Co., Tokyo, Japan; that contained 96.6% cellobiose β 1–4, 1.9% cello-oligosaccharides, 1.5% glucose, and no nitrogen content). These concentrations were selected to study a lower dose of cellobiose than the optimal found previously (7.5 g/L). Two experimental diets were formulated to differ in their dietary soluble fibre concentration (71.9 and 130 g/kg DM, for the low soluble fibre (LSF) and high soluble fibre (HSF) diets, respectively) and starch (222 and 184 g/kg DM for LSF and HSF, respectively). It resulted in six treatments (LSF0, LSF3.5, LSF7, HSF0, HSF3.5, and HSF7). Ingredients and chemical composition of diets are shown in Table 2.

A total of 192 crossbred mixed-sex rabbits (New Zealand White $\times$ Californian, V $\times$ R from UPV, Valencia, Spain) were weaned at 26 d of age, with a BW of 457 ± 67 g, blocked by litter, and randomly assigned to the six treatments (32 rabbits/treatment). They were housed individually and had ad libitum access to feed and water over the trial and received no antibiotic. The feed intake, weight gain and mortality were recorded individually. The water intake was estimated according to the water/feed intake ratio obtained with these two diets by Delgado et al. (2015). At 39 d of age, experimental diets were replaced by a standard commercial diet (g/kg DM: 172 CP, 338 NDF and 60.2 soluble fibre), continuing with the cellobiose supplementation in water until 42 d of age to limit the potential negative effects of removing simultaneously the experimental diet and the cellobiose. The experiment finished at 56 d of age. Before weaning rabbits had access to their mothers' feed (g/kg DM: 180 CP, 329 NDF, and 93.4 soluble fibre).

Another, 48 young rabbits were weaned at 26 d of age, with a BW of 475 ± 74 g, blocked by litter, and randomly assigned to one of the six treatments (8/treatment).

Table 2. Ingredient and chemical composition of the experimental diets.
(Experiments 2, 3 and 4)

	Low soluble fibre (LSF)	High soluble fibre (HSF)
Ingredient, g/kg as fed basis		
Wheat bran	280	130
Wheat straw	100	50.0
Beet pulp	0.00	180
Sunflower meal 29	99.7	130
Dehydrated alfalfa 13	5.0	150
Soybean meal 44	80.0	80.0
Wheat grain	227	217
Sunflower oil	20.0	20.0
Linseed oil	10.0	10.0
L-Lysine HCl	4.40	4.40
DL-Methionine	0.80	0.60
L-Threonine	3.10	3.20
Calcium carbonate	12.0	7.0
Sodium chloride	3.0	3.10
Calcium phosphate	5.0	10.0
Vitamin/mineral premix ¹	5.0	5.0
Chemical composition, g/kg DM		
DM	906	910
Ash	72.9	67.2
CP	164	165
Total dietary fibre (TDF)	380	438
NDF ²	309	308
ADF ³	167	187
ADL ³	31.0	34.0
Soluble fibre	71.9	130
Starch	222	184
Ether extract	49.1	50.0
Sugars	82.3	84.4

¹ Provided by Trouw Nutrition (Madrid, Spain). Mineral and vitamin composition (per kg of complete diet): 20 mg of Mn as MnO; 59.2 mg of Zn as ZnO; 10 mg of Cu as CuSO₄ 5H₂O; 1.25 mg of I as KI; 0.495 mg of Co as CoCO₃ H₂O H₂O; 76 mg of Fe as FeCO₃; 8375 UI of vitamin A; 750 UI of vitamin D₃, 20 UI of vitamin E as DL- α -tocopherol acetate, 1.0 mg of vitamin K; 1.0 mg of vitamin B₁; 2 mg of vitamin B₂; 1 mg of vitamin B₆; 20 mg of Niacin; 54.1 mg of Betaine; 137.5 mg of Choline chloride; 66 mg of robenidine; 50 mg of ethoxyquin. ² Values were corrected for ash and crude protein. ³ Values were corrected for ash.

They were housed collectively and they had ad libitum access to water supplemented with the three concentrations of cellobiose (0.0, 3.5 and 7.0 g/L) and the two diets differing in the soluble fibre level (LSF and HSF, respectively). After 13 d adaptation period, and with an average BW of 1075 ± 153 g, they were slaughtered by head concussion between 19:00 and 21:00 h. After slaughter, the whole gastrointestinal tract was removed and weighed. The caecum with digesta, stomach, and liver were weighed individually. In addition, 6 cm samples were excised from the middle part of the jejunum, flushed with saline solution, frozen in dry ice, and immediately stored at -20°C to determine sucrose activity as described by Gómez-Conde et al. (2007).

3.1.1.3. Experiment 3: “Effect of cellobiose supplementation on growth performance in rabbits fed a low soluble fibre diet”.

Three cellobiose concentrations in drinking water (0.0, 3.5, and 7.0 g/L) were offered to rabbits fed the LSF diet (71.9 g soluble fibre/kg DM) (same used in experiment 2) to confirm the previous results. A total of 186 crossbred mixed-sex rabbits (New Zealand White $\times$ Californian, V $\times$ R from UPV, Valencia, Spain) were weaned at 26 d of age, with a BW of 447 ± 65 g, blocked by litter, and randomly assigned to the three treatments (62/treatment). They were housed individually and had ad libitum access to feed and water over the trial and received no antibiotic. The feed intake, weight gain and mortality were recorded individually. The water intake was estimated according to Delgado et al. (2015). At 40 d of age, LSF diet was replaced by a standard commercial diet (g/kg DM: 172 of CP, 338 of NDF and 60.2 of soluble fibre), continuing with the supplement of cellobiose in water until 50 d of age. The experiment finished at 61 d of age. Before weaning rabbits had access to their mothers' feed (the same than in experiment 2).

3.1.1.4. Experiment 4: “Effect of pre- and post-weaning cellobiose supplementation on growth performance and health in rabbits”.

Pre-weaning period. Two cellobiose concentrations in drinking water free of antibiotics (0.0 and 7.0 g cellobiose/L, preW_0.0 and preW_7.0, respectively) and a standard commercial diet (g/kg DM: 164 CP, 341 NDF, and 58.7 soluble fibre) were

provided to suckling rabbits. A total of 20 litters (10 kits/litter with an average individual BW of 353 ± 27.3 g/rabbit) from multiparous rabbits does were used. At 22 d of age each litter was divided in two groups (5 kits/group), and randomly assigned to one of the two treatments (preW_0.0 and preW_7.0; $n = 20$ litters/treatment). They were caged collectively and had ad libitum access to a standard commercial feed (g/kg DM: 164 CP, 341 NDF, and 58.7 soluble fibre), and water over the trial and received no antibiotic. The feed intake and weight gain were recorded collectively. From 22 to 29 d of age (pre-weaning period) young rabbits were taken daily to their mother cage for suckling (10 min). Before the separation from their mothers, rabbits had access to their mothers' feed (the same than in experiment 2).

Post-weaning period. At weaning (29 d), four rabbits per litter of each group (preW_0.0 and preW_7.0) chosen at random were divided in two groups (2 rabbits/litter and treatment), caged individually and assigned the one of the two doses of cellobiose (0.0 and 7.0 g cellobiose/L, postW_0.0 and postW_7.0, respectively). It resulted in four experimental groups (40/group): [preW_0.0- postW_0.0], [preW_0.0- postW_7.0], [preW_7.0-postW_0.0], and [preW_7.0-postW_7.0]. At 50 d of age the cellobiose supplementation was suspended and the trial finished at 57 d of age. The remaining 20 weaned rabbits per group (preW_0.0 and preW_7.0) were also divided in two groups (10 rabbits/treatment), caged individually and assigned the one of the two doses of cellobiose (0.0 and 7.0 g cellobiose/L, postW_0.0 and postW_7.0, respectively). It resulted in four experimental groups (10/group). These rabbits were slaughtered at 39 d of age and the weight of digestive tract and liver and caecal pH were recorded.

2.1.2. Objective 2:

Evaluate the influence of cellobiose and soluble fibre supplementation on the *in vitro* caecal fermentation of cellobiose and other carbohydrates-rich feedstuffs.

In order to reach this objective two experiments were done.

3.1.2.1. Experiment 5: “*In vitro* caecal fermentation of carbohydrate-rich feedstuffs in rabbits as affected by substrate pre-digestion and donors’ diet”.

Eight hybrid rabbits (New Zealand White x Californian) were weaned at 34 d of age and assigned randomly to each of the two experimental diets (4 rabbits/diet). The experimental diets were formulated to differ in their content of soluble fibre mainly provided by sugar beet pulp (84.0 and 130 g/kg for the low soluble fibre (LSF) and high soluble fibre (HSF) diets, respectively; dry matter (DM) basis) and starch (226 and 182 g/kg DM for LSF and HSF, respectively). Ingredients and chemical composition of diets are given in Table 1. Rabbits had ad libitum access to feed and fresh water over the trial and received no antibiotic. At 41 d of age (1.13 ± 0.005 kg body weight) rabbits were fitted with plastic collars from 9:00 to 10:00 h (maximum) to collect the soft faeces to be used as inoculum for the *in vitro* incubations. As soon as they were produced, the soft faeces were wrapped in aluminium foil to reduce air contact and thermal shock, and were immediately transported to the laboratory into thermal flasks.

Four fibrous or fibre-derived, low-starch, and low-protein ingredients, having a wide range of fermentation rate and extent were used as substrates for the *in vitro* incubations: D-cellobiose (CEL; NPC Cello-Oligo, Nippon Paper Industries Co., Tokyo, Japan), sugar beet pulp (SBP; Fipec, Nordic Sugar, Copenhagen, Denmark), sugar beet pulp pectin (PEC; Betapec RU 301, Herbstreith & Fox, Neuenbürg, Germany) and wheat straw (WS; Pagran, PITE S.A., Tordesillas, Spain). Most of chemical constituents in all ingredients selected for this study cannot be hydrolysed by endogenous enzymes of adult rabbits. According to the manufacturer, CEL contained 96.6% cellobiose β 1–4, 1.9% cello-oligosaccharide, 1.5% glucose, and no nitrogen content. The SBP had 646 g total dietary fibre (TDF), 369 g neutral detergent fibre (NDF), 86.3 g crude protein (CP), and 39.4 CP-TDF/kg DM, PEC had 934 g TDF, 6.4 g NDF, 53.1 g CP and 16.2 g CP-TDF/kg DM, and WS

contained 785 g TDF, 748 g NDF, 28.0 g CP and 27.9 g CP-TDF/kg DM. All TDF and NDF values were corrected for their ash and CP content.

Samples (250 mg DM) of each substrate were carefully weighed into 115-ml glass vials. In half of the vials, the substrates were subjected to a two-step pepsin-pancreatin *in vitro* digestion (pre-digestion) according to Ramos et al. (1992), with the exception that the contents of the vials were not filtrated at the end of the procedure. All vials were stored at 4°C overnight (to stop the digestion) and placed in an incubator at 40°C for 1 h before starting the *in vitro* incubations. A total of 64 vials with substrate (2 pre-digestion $\times$ 2 donors' diet $\times$ 4 substrates $\times$ 4 rabbits/diet) were incubated. In addition, 16 vials without substrate (blanks; two per rabbit) were prepared and half of them underwent the pre-digestion procedure.

For the *in vitro* incubations, soft faeces from each rabbit were mixed with the culture medium described by Goering and Van Soest (1970) and homogenized with a blender for 2 min. The soft faeces/medium ratio (720 mg/100 ml) was selected from previous studies by our group (it was selected from concentrations ranging from 415 to 2000 mg/100 ml. unpublished results). Vials were filled up with 25 ml of the mixture using a Watson-Marlow 520UIP31 peristaltic pump (Watson-Marlow Fluid Technology Group, Cornwall, United Kingdom), sealed with rubber stoppers, and incubated at 40°C for 144 h. This temperature (40°C) was set up in the incubator to reach 39°C in the content of the glass vials used for the incubations, consistently with the internal body temperature of rabbits. This long incubation time was selected to reach the potential degradation in all samples. The preparation of the medium, its mixture with the soft faeces, and vials filling were carried out under continuous CO₂ flushing at 40°C. Gas production was measured at 4, 6, 9, 12, 20, 24, 30, 35, 48, 58, 72, 96, 120 and 144 h using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL, Barcelona, Spain) and a plastic syringe, and the gas produced at each measurement time was released. Immediately after measuring the gas production at 24 h, 1 ml of each vial content was taken using an insulin syringe, mixed with 20 μ l of H₂SO₄ (10% vol/vol) and stored at -20°C for VFA analysis. Samples for VFA analysis were thawed and processed as previously described (experiment 1).

3.1.2.2. Experiment 6: “Effect of cellobiose supplementation and dietary soluble fibre content on *in vitro* caecal fermentation of carbohydrate-rich substrates in rabbits”.

Six experimental treatments in a 3×2 factorial arrangement, with 3 cellobiose concentrations in drinking water and 2 dietary levels of soluble fibre, were used. The three concentrations of cellobiose were 0.0, 7.5 and 15.0 g/L, and were obtained by diluting D-cellobiose (NPC Cello-Oligo, Nippon Paper Industries Co., Tokyo, Japan) in the drinking water free of antibiotics. According to the manufacturer, D-cellobiose contained 96.6% cellobiose β 1–4, 1.9% cello-oligosaccharide, 1.5% glucose, and no nitrogen content. These concentrations were selected to obtain a wider range of cellobiose supplementation than those recommended for other poultry and pigs (0.15 - 0.45% in the diet. Jiao et al., 2014; Song et al., 2013).

Two experimental diets free of antibiotics, were formulated to differ in the concentration of both soluble fibre (84.0 and 130 g/kg DM for the low (LSF) and high soluble fibre (HSF) diets, respectively) and starch (226 and 182 g/kg DM for LSF and HSF diets, respectively). This experimental design resulted in six dietary treatments named LSF0, LSF7.5, LSF15, HSF0, HSF7.5 and HSF15. Ingredients and chemical composition of diets are shown in Table 1.

Twenty four hybrid rabbits (New Zealand White x Californian) were weaned at 34 d of age and assigned randomly to each of the six experimental treatments (four rabbits / treatment). Rabbits had ad libitum access to feed and fresh water over the trial, and received no antibiotic treatment. At 41 d of age (1.13 ± 0.005 kg body weight) all rabbits were fitted with plastic collars from 9:00 to 10:00 h (maximum) to collect the soft faeces to be used as inoculum for the *in vitro* incubations. The soft faeces were wrapped in aluminum foil to reduce air contact, and were immediately transported to the laboratory into thermal flasks.

Five fibrous (or derived from fibrous) ingredients were selected as substrates for the *in vitro* incubations: D-cellobiose (CEL, NPC Cello-Oligo, Nippon Paper Industries Co., Tokyo, Japan; according to the manufacturer contained 96.6% cellobiose β 1–4, 1.9% cello-oligosaccharide, and 1.5% glucose), D-(+)-glucose (GLU, Sigma n. 8270), sugar beet pulp (SBP, Fipec, Nordic Sugar, Copenhagen,

Denmark; contained 646 g total dietary fibre (TDF) and 369 g aNDFom/kg DM; both values corrected for ash and crude protein), SBP pectin (PEC, Betapec RU 301, Herbstreith & Fox, Neuenbürg, Germany), and wheat straw (WS, Pagan, PITE S.A., Tordesillas, Spain; contained 785 g TDF and 748 g NDF/kg DM). All substrates have low content in starch and protein, and were expected to have a wide range of fermentation rate and extent. Thus, most of chemical constituents of CEL, SBP, PEC and WS cannot be digested by endogenous enzymes and absorbed by rabbits, but GLU is a rapid and completely fermentable substrate.

Samples (250 mg DM) of each substrate were carefully weighed into 115-ml glass vials and were subjected to a two-step pepsin-pancreatin *in vitro* digestion (pre-digestion) following the method of Ramos et al. (1992), with the only exception that the contents of the vials were not filtrated at the end of the procedure. All vials were stored at 4°C overnight to stop the digestion process, and placed back in an incubator at 40°C for 1 h before starting the *in vitro* incubations. A total of 120 vials with substrate (3 cellobiose concentrations $\times$ 2 donors' diet $\times$ 5 substrates $\times$ 4 rabbits / cellobiose concentration $\times$ diet) and 24 vials without substrate (blanks; one per rabbit) were pre-digested before *in vitro* incubations.

The *in vitro* incubations, times of incubations and sampling of VFA were done followed the procedure explained in experiment 5. Samples for VFA analysis were thawed and processed as previously described (experiment 1).

3.2. CHEMICAL ANALYSIS

Procedures of the AOAC (2000) were used to determine the concentrations of DM (934.01), ash (967.05), CP (968.06), ether extract (920.39), starch (amyloglucosidase- α -amylase method, 996.11) and total dietary fibre (985.29. TDF). Dietary NDF was determined using the filter bag system (Ankom Technology, New York) according to Mertens et al. (2002), and a thermo-stable amylase without any sodium sulphite added. Values were corrected for ash and protein. Dietary ADF and ADL were analyzed according to AOAC (2000; method 973.187) and Goering and Van Soest (1970), respectively. The soluble fibre content was calculated by difference as TDF–NDF. Sugars were analyzed according to Yemm and Willis (1954).

3.3. STATISTICAL ANALYSIS

Growth trail results from Exps. 1 and 2 and VFA concentrations from Exp.1 were analyzed by using a mixed model (PROC MIXED, SAS Inst. Inc., Cary, NC) that included as fixed factors the level of soluble fibre and cellobiose and their interaction, and the litter as a random effect. Weaning weight was included as a linear covariate. Mortality was analyzed using a logistic regression (GENMOD procedure of SAS considering a binomial distribution) including the level of soluble fibre and cellobiose and their interaction in the model, and the results were transformed from the logit scale. Non-orthogonal contrasts were used to compare the LSF and HSF groups with no cellobiose supplementation.

The analysis of gene expression in Exp. 1 was determined using a mixed-model in which the levels of soluble fibre and cellobiose were included as fixed factors and the sample as a random effect (Steibel et al., 2009). For genes displaying efficiencies different from 2 ($E \neq 2$), Ct values were adjusted according to the model described by Steibel et al. (2009). The standard error (SE) was used to recalculate the lower and upper 95% confidence intervals for each fold change. In all cases, linear and quadratic polynomial contrasts were used to test the linear and quadratic effects of the level of cellobiose and their interactions with the level of soluble fibre. When any interaction between cellobiose and dietary fibre were significant, specific linear and quadratic contrasts to study the effect of cellobiose were done within each level of soluble fibre. Statistical analysis of experiment 3 was the same than in experiment 1 and 2 but excluding the effect of soluble fibre.

In experiment 4, pre-weaning growth traits were analysed by using a mixed model including the cellobiose concentration in the model. Initial weight at 22 d was included as a linear covariate and the litter as a random effect. Post-weaning growth traits were analysed as a 2×2 factorial arrangement (2 periods $\times$ 2 cellobiose doses), weaning weight was included as a linear covariate and the litter as a random effect. Mortality was analysed using a logistic regression (GENMOD procedure of SAS considering a binomial distribution).

For the *in vitro* data (Exps. 5 and 6), the values of gas produced at each measurement time and those of VFA at 24 h were corrected for the amount of gas

and VFA, respectively, produced in the corresponding blanks. Gas production data were fitted to the logistic model described by Schofield et al. (1994):

$$\text{Gas production} = V_f / [1 + e^{2 - 4k(t - \text{Lag time})}]$$

where V_f is the final asymptotic gas production, k is the fractional rate of gas production, *Lag time* is the initial delay in the onset of gas production (lag time) and t is the time of gas measurement. The V_f , k and lag time parameters were estimated by an iterative least squares procedure (Marquardt algorithm) using the NLIN procedure of SAS (version 9.2; SAS Inst. Inc., Cary, NC, USA). The maximum gas production rate (μ_m) and the time when μ_m is reached (T_i) were calculated according with Schofield et al. (1994) as $\mu_m = k \times V_f$ and $T_i = \text{Lag time} + (V_f / (2 \times \mu_m))$.

Data on gas production parameters and VFA production of experiment 5 were analysed as a mixed model including the substrate processing (with or without pre-digestion procedure), the diet of donor rabbits (LSF and HSF), the type of substrate and their interactions as fixed effects, and donor rabbits (inoculum) as a random effect.

The gas production parameters and VFA production data of experiment 6 were analysed as a mixed model including the cellobiose concentration (0.00, 7.50 and 15.0 g / L), diet of donor rabbits (LSF and HSF) and substrate as fixed effects and donor rabbits (inoculum) as a random effect. Linear and quadratic polynomial contrasts were developed to study the linear and quadratic effects of the level of cellobiose and their interactions with the level of soluble fibre.

For experiments 5 and 6, when a significant effect of substrate was detected ($P < 0.05$), the Tukey test was used for mean comparisons. Significance was declared at $P < 0.05$, whereas $P < 0.10$ values were considered to be a trend.

IV. RESULTS

4.1. OBJECTIVE 1:

Determine the adequate dose of cellobiose and its potential interaction with the dietary soluble fibre level. Characterize the ileal and caecal fermentative profile of the cellobiose when combined with different levels of soluble fibre.

4.1.1. Experiment 1: “The effect of cellobiose on the health status of growing rabbits depends on the dietary level of soluble fibre”.

The ADFI from 34 to 48 d of age decreased by 9% in the LSF compared to the HSF-fed group (109 vs. 102 g/d; $P < 0.001$; Table 3), but no differences were found either in ADG or G:F ($P \geq 0.12$). The level of cellobiose had a quadratic effect on ADFI and ADG in this period, showing the 7.5-cellobiose groups the highest values (108 vs. 102 g/d, and 52.7 vs. 47.9 g/d, respectively; $P \leq 0.031$). This led to a quadratic effect of cellobiose on G:F ($P = 0.047$), with the 7.5-cellobiose group having the highest values (0.487 vs. 0.460, averaged value for 0 and 15.0 cellobiose). No interaction ($P > 0.05$) between the level of soluble fibre and cellobiose was found for growth traits. Estimations of water and cellobiose intake are show in Table 3. The supplementation with 7.5 and 15 g cellobiose/L drinking water was estimated to be equivalent to a dietary level of inclusion of cellobiose of 1.1 and 2.2% (as fed) for LSF group, and to 1.3 and 2.6% for HSF group, respectively. Once the cellobiose was withdrawn (at 48 d of age) and all rabbits received a standard diet, less difference among groups were observed. A trend ($P = 0.063$) to an interaction between the quadratic effect of cellobiose and the level of soluble fibre affected G:F ratio.

The effects of the experimental factors in the whole fattening period were similar to those observed from 34 to 48 d of age. The high level of soluble fibre impaired ADFI by 5% (140 vs. 133 g/d; $P = 0.014$) and tended to reduce ADG ($P = 0.066$) with no influence on G:F. Cellobiose supplementation had no influence on G:F in the LSF-fed group but exerted a quadratic effect in the HSF group ($P = 0.003$), and a trend to an interaction between the quadratic effect of cellobiose supplementation and the level of soluble fibre ($P = 0.083$) was detected.

Table 3. Effect of the level of cellobiose in drinking water and dietary soluble fibre on the growth performance of rabbits. (*Experiment 1*)

Soluble fibre	Low			High			SEM	P- value					
Cellobiose, g/L	0.00	7.50	15.0	0.00	7.50	15.0		Cov ²	Soluble fibre	Cellobiose		Cellobiose × soluble fibre	
n ¹	26	32	29	33	30	27				Lineal	Quadratic	Lineal	Quadratic
34-48 d ³													
BW at 34 d, g	768	794	795	791	771	770	21.0	-	0.55	0.86	0.92	0.15	0.43
ADFI, g/d	111	112	104	98.9	104	95.9	3.20	<0.001	<0.001	0.13	0.031	0.54	0.72
ADG, g/d	49.9	54.5	48.3	47.6	51.0	45.8	2.18	0.31	0.12	0.45	0.010	0.95	0.77
G:F, g/g	0.449	0.487	0.452	0.471	0.487	0.470	0.016	<0.001	0.28	0.95	0.047	0.89	0.43
Water intake, g/d ⁴	166	168	156	173	183	168	5.21	-	-	-	-	-	-
Cellobiose intake, g/d	0.00	1.26	2.34	0.00	1.37	2.51	0.053	-	-	-	-	-	-
Mortality, %	25.7	5.71	17.1	0	5.71	17.1	-	-	0.020	0.029	0.84	0.003	0.087
48-61d ⁵													
BW at 48 d, g	1481	1546	1460	1449	1497	1423	30.6	<0.001	0.12	0.45	0.010	0.95	0.77
ADFI, g/d	175	171	175	166	164	175	5.40	0.071	0.23	0.40	0.27	0.44	0.79
ADG, g/d	48.6	44.6	47.5	45.9	46.2	43.9	2.13	0.73	0.37	0.47	0.56	0.82	0.19
G:F, g/g	0.279	0.247	0.276	0.276	0.284	0.221	0.020	0.17	0.67	0.16	0.89	0.21	0.063
Mortality, %	0.00	2.86	0.00	5.71	8.57	5.71	-	-	0.010	1.00	0.12	1:00	0.23
34-61d													
BW at 61 d, g	2113	2126	2077	2046	2097	1993	39.6	<0.001	0.066	0.28	0.11	0.83	0.49
ADFI, g/d	141	140	138	131	133	134	3.56	<0.001	0.014	0.90	0.81	0.39	0.99
ADG, g/d	49.3	49.7	47.9	46.8	48.7	44.8	1.47	0.30	0.066	0.28	0.11	0.83	0.49
G:F, g/g	0.350	0.354	0.349	0.356	0.367	0.328	0.0066	<0.001	0.97	0.034	0.011	0.050	0.083
Mortality, %	25.7	8.57	17.1	5.71	14.3	22.9	-	-	0.53	0.28	0.36	0.029	0.16

¹ n = number of rabbits at the end of the fattening period and used to calculate growth traits. For mortality values the initial number of rabbits was 35/treatment. ² Live weight at weaning was used as covariate. ³ Period in which rabbits were supplemented cellobiose in drinking water and fed the two experimental diets (low soluble fibre 71.9 g/kg DM; high soluble fibre 130 g/kg DM). ⁴ Estimated according to Delgado et al. (2015) that obtained a ratio water to feed intake of 1.50 and 1.75 for LSF and HSF diets, respectively. ⁵ Period in which both cellulose and experimental diets were withdrawn and rabbits received a standard feed and no additive in water.

Mortality rate was influenced both by cellobiose and soluble fibre levels and there were linear and quadratic interactions between the two factors (Table 3). From 34 to 48 d of age there was a linear increase of mortality with increasing cellobiose levels in the HSF group (from 0 to 17.1%; $P = 0.003$), and this effect remained when the whole fattening period was considered ($P = 0.035$). In contrast, a quadratic effect of the level of cellobiose on mortality was found in the LSF group, showing the rabbits offered 7.5-cellobiose the lowest mortality from 34 to 48 d of age (5.7 vs. 21.4%, averaged value for 0 and 15.0 cellobiose; $P = 0.030$), and this effect tended to be observed when the whole fattening period was considered ($P = 0.091$). The mortality of rabbits that received no cellobiose was lower with the HSF than with the LSF diet both from 34 to 48 d (0 vs. 25.7; $P = 0.030$) and in the whole fattening period (5.7 vs. 25.7%; $P = 0.017$). However, when rabbits were fed a standard diet (from 48 to 61 d) the mortality was higher in the HSF compared with the LSF group, indicating that mortality occurred later (but in a lower proportion) in HSF than in LSF rabbits. The accumulated mortality revealed a different evolution of mortality among rabbits from different treatments (Figure 1). Once cellobiose and the experimental diets were withdrawn, mortality increased in rabbits previously fed the HSF diet, with no changes in those fed the LSF diet.

A cellobiose $\times$ soluble fibre interaction ($P = 0.023$) was observed for the sucrose activity in the jejunal mucosa, as the 7.5 cellobiose group showed a maximal value with the LSF diet (232 vs. 191 $\mu\text{mol glucose/g protein}$) but a minimal value with the HSF diet (187 vs. 229 $\mu\text{mol glucose/g protein}$) compared with the 0 or 15.0 cellobiose groups (Table 4). The relative weight of the digestive tract was not influenced by the level of soluble fibre ($P = 0.51$), but tended to increase quadratically with increasing cellobiose levels ($P = 0.071$; Table 4). There was no effect of treatments on the relative weight of the empty caecum or caecal digesta ($P \geq 0.18$). The experimental treatments had no influence on ileal pH ($P > 0.26$), but an interaction between the level of cellobiose and that of soluble fibre was found for caecal pH ($P = 0.045$; Table 5). The 7.5 cellobiose group had the highest caecal pH values when the LSF diet was fed (5.50 vs. 5.36, averaged value for 0 and 15.0 g/L cellobiose), but the lowest values with the diet HSF (5.26 vs. 5.36). The pH was

Figure 1. Effect of the level of cellobiose in drinking water (0, 7.5 and 15.0 g/L) and dietary soluble fibre content (low: LSF 84.0 g/kg DM; high: HSF 130 g/kg DM) on the accumulated mortality of rabbits from weaning to 61 d of age. The vertical line at 48 d of age indicates the end of experimental treatments (cellobiose and diets) and the change to a common commercial diet to all rabbits. (*Experiment1*)

higher in the ileum than in the caecum ($P < 0.001$) for all treatments, and caecal digesta had about two times more DM content than the ileal one ($P < 0.001$). The DM content of both ileal and caecal digesta was not influenced by cellobiose level, but caecal DM content decreased with the level of soluble fibre (20.9 vs. 19.4%; $P = 0.031$).

Both ileal and caecal total VFA concentrations were higher (22 and 11%, respectively; $P \leq 0.005$) in HSF-fed rabbits than in those fed the LSF diet (Table 5). The level of cellobiose influenced quadratically the ileal total VFA concentrations, with the 7.5-cellobiose group showing the maximal value (26.3 vs. 23.3 mmol/g fresh digesta; $P = 0.014$). In the caecum, total VFA concentrations decreased linearly with the level of cellobiose in HSF-fed rabbits ($P = 0.006$), whereas no effect was observed in LSF groups, resulting in an interaction between the level of cellobiose and the level of soluble fibre ($P = 0.033$). Total VFA concentrations were higher in caecal than in ileal digesta (87.5 vs. 24.3 mmol/g fresh digesta; values averaged across experimental treatments; $P < 0.001$; Table 4). Caecal VFA concentrations was negatively correlated to mortality from 34 to 48 d of age ($r = -0.89$; $P = 0.018$; $n = 6$) and a trend was found for the mortality in the whole fattening period ($r = -0.77$; $P = 0.076$; $n = 6$).

The VFA profile in the ileal and caecal digesta was influenced by both cellobiose and soluble fibre levels. In the ileal digesta, acetate and butyrate proportions were affected quadratically by the cellobiose level in LSF-fed rabbits, with no effect in HSF-fed rabbits, which resulted in a trend to an interaction cellobiose $\times$ soluble fibre ($P \leq 0.086$; Table 4). A similar result was found for propionate proportions, but no cellobiose $\times$ soluble fibre interaction was observed ($P = 0.19$). The rabbits fed the LSF diet and supplemented with 7.5-cellobiose had lower acetate and greater propionate and butyrate proportions in the ileum compared with those on 0 and 15.0 cellobiose treatments. In contrast, no effects of cellobiose supplementation on the main VFA proportions were observed in rabbits fed HSF diet, but caproate proportion tended to decrease linearly with cellobiose inclusion ($P = 0.070$). A negative correlation between ileal caproate concentration and mortality rate in the whole fattening period was observed ($r = -0.86$; $P = 0.019$; $n = 6$).

Table 4. Effect of the level of cellobiose in drinking water and dietary soluble fibre on digestive traits in 46 d old rabbits. (*Experiment1*)

Soluble fibre Cellobiose, g/L n ¹	Low			High			SEM	<i>P- value</i>				
	0	0.75	1.5	0	0.75	1.5		Soluble fibre	Cellobiose		Soluble fibre × Cellobiose	
	7	9	8	9	8	9			Lineal	Quadratic	Lineal	Quadratic
BW, g	1231	1316	1311	1308	1353	1296	60.70	0.51	0.58	0.36	0.46	0.95
Sucrose activity in jejunal mucosa, µmol glucose/ g protein	200	232	182	212	187	227	17.1	0.79	0.94	0.76	0.84	0.023
Weight of the digestive tract, % BW	28.3	27.8	28.9	28.8	26.9	29.6	0.97	0.91	0.48	0.071	0.95	0.41
Caecum												
Empty weight, % BW	2.52	2.54	2.71	2.51	2.44	2.74	0.15	0.84	0.18	0.32	0.87	0.69
Digesta, % BW	8.57	7.65	8.35	7.82	8.34	8.75	0.56	0.81	0.53	0.44	0.31	0.38

¹ n= number of rabbits. Low and high soluble fibre content in the diet (84.0 and 130 g/kg DM, respectively).

Table 5. Effect of the level of cellobiose in drinking water and dietary soluble fibre on the total and molar VFA at ileal and caecal concentration in 46 d old rabbits. (*Experiment 1*)

Soluble fibre	Low			High			SEM	<i>P- value</i>				
Cellobiose, g/L	0.00	7.50	15.0	0.00	7.50	15.0		Soluble fibre	Cellobiose		Soluble fibre × Cellobiose	
n ¹	7	8	6	8	8	8			Lineal	Quadratic	Lineal	Quadratic
n ²	7	8	8	9	8	9						
Ileum												
DM, %	10.5	12.9	11.4	10.5	10.7	10.7	0.97	0.23	0.57	0.21	0.74	0.25
pH	6.91	6.84	6.99	6.90	6.97	6.97	0.065	0.59	0.27	0.49	0.88	0.21
Total VFA, mmol/g fresh digesta	19.8	25.1	20.9	25.9	27.6	26.5	1.41	<0.001	0.56	0.014	0.85	0.18
Molar proportion, mol/100 mol												
Acetate	93.9	91.2	95.4	93.6	94.0	95.0	0.91	0.34	0.14	0.019	0.94	0.043
Propionate	4.37	5.65	2.67	3.70	4.38	3.46	0.58	0.42	0.11	0.005	0.23	0.19
Butyrate	1.49	2.44	1.45	1.89	1.34	1.41	0.42	0.48	0.56	0.36	0.61	0.086
Isobutyrate	0.00	0.096	0.015	0.040	0.00	0.008	0.046	0.58	0.85	0.41	0.61	0.16
Valerate	0.024	0.096	0.027	0.18	0.004	0.049	0.058	0.56	0.29	0.69	0.26	0.075
Isovalerate	0.091	0.068	0.22	0.18	0.080	0.085	0.050	0.79	0.75	0.11	0.040	0.68
Caproate	0.11	0.50	0.23	0.40	0.17	0.040	0.14	0.50	0.41	0.25	0.11	0.13
Caecum												
DM, %	19.5	20.9	22.4	19.2	19.9	19.0	0.86	0.031	0.13	0.64	0.086	0.57
pH	5.37	5.50	5.35	5.39	5.26	5.33	0.067	0.17	0.57	0.72	0.82	0.045
Total VFA, mmol/g fresh digesta	81.0	86.7	82.9	99.5	94.5	84.5	3.81	0.005	0.092	0.28	0.033	0.74
Molar proportion, mol/100 mol												
Acetate	83.5	83.4	82.5	84.2	87.1	84.8	1.27	0.036	0.83	0.18	0.53	0.31
Propionate	2.85	3.29	2.30	2.94	2.58	3.02	0.40	0.92	0.56	0.66	0.44	0.12
Butyrate	11.8	11.6	13.5	11.9	9.43	11.2	0.97	0.076	0.60	0.066	0.23	0.52
Isobutyrate	0.017	0.094	0.011	0.027	0.00	0.00	0.017	0.024	0.33	0.027	0.52	0.002
Valerate	0.26	0.24	0.20	0.20	0.12	0.15	0.035	0.011	0.13	0.52	0.78	0.26
Isovalerate	0.15	0.20	0.18	0.10	0.060	0.15	0.022	<0.001	0.095	0.43	0.63	0.010
Caproate	1.38	1.22	1.37	0.60	0.65	0.70	0.20	<0.001	0.84	0.66	0.79	0.64

¹ number of ileal VFA samples. ² number of caecal VFA samples. Low and high soluble fibre content in the diet (84.0 and 130 g/kg DM, respectively).

Acetate proportions were higher and those of butyrate, isobutyrate, valerate, isovalerate and caproate were lower in the caecal digesta of rabbits fed the HSF diet compared with LSF-fed rabbits ($P \leq 0.076$). Cellobiose supplementation had less influence on VFA proportions in the caecum than in the ileum. Cellobiose influenced quadratically ($P \leq 0.066$) the proportions of butyrate in both LSF and HSF rabbits (minimal values for 7.5 cellobiose groups), isobutyrate in LSF-fed rabbits (maximal values for 7.5 cellobiose group) and isovalerate in HSF rabbits (minimal values for 7.5 cellobiose group). The VFA profile differed between the ileal and caecal digesta ($P < 0.001$), with the exception of isobutyrate and isovalerate proportions. Ileal digesta had higher ($P < 0.001$) proportions of acetate (93.8 vs. 84.4%; values averaged across treatments) and propionate (4.04 vs. 2.82) than caecal digesta, but lower ($P < 0.001$) proportions of butyrate (1.67 vs. 11.5%), valerate (0.06 vs. 0.19%) and caproate (0.24 vs. 0.98%).

The relative gene expression of all studied cytokines (IL-6, IL-10, and TNF- α), iNOS, MUC-1 and toll like receptors (TLR-2 and TLR-4) in the appendix increased linearly and quadratically with the level of cellobiose ($P \leq 0.063$; Fig. 2), resulting in higher gene expression for HSF than for LSF-fed rabbits when combined with 15.0 cellobiose ($P \leq 0.058$), except for iNOS. The relative gene expression of MUC-13 was not influenced by the experimental treatments ($P > 0.10$).

Figure 2. Effect of the level of cellobiose in drinking water (0, 7.5 and 15 g/L) and dietary soluble fibre in the diet (low 84.0 and high 130 g/kg DM, respectively) on mRNA levels interleukin 6 and 10 (IL-6, IL-10), inducible nitric oxide synthase (iNOS), MUC-1, TLR-2, TLR-4 and tumor necrosis factor- α (TNF α). Relative gene expression values are fold change of 7.5- and 15-cellobiose groups relative to the 0-cellobiose (control), for each level of fibre. Bars indicate the 95% confidence interval (Fold change up-Fold change low). n = 8/treatment. (**: P < 0.01; ***: P < 0.001). Lower case letters indicate differences of expression among cellobiose levels. (*Experiment1*)

4.1.2. Experiment 2: “Effect of cellobiose supplementation on growth performance and health in rabbits”.

Dietary soluble fibre along the experiment did not affect the mortality rate (Table 6 and Figure 3. $P \geq 0.15$). However, when no cellobiose was supplemented the increase of dietary soluble fibre reduced the mortality from 26 to 39 d of age (25.0 vs. 6.3%. $P = 0.033$), although this effect disappeared at the end of the experiment. The supplementation of cellobiose tended to reduce linearly the mortality from 26 to 39 d ($P = 0.11$), and in the whole experimental period ($P = 0.098$), mainly due to the linear reduction of mortality on LSF fed groups in both periods ($P \leq 0.026$). On the opposite, mortality rate evolved quadratically in HSF groups, and it tended to decrease in the HSF3.5 group from 39 to 56 d of age and in the whole experimental period ($P \leq 0.091$). Mortality in LSF0 group occurred mainly two weeks after weaning with a minor rise in the second phase of fattening (Figure 3), differing greatly from mortality in HSF0 group that was observed mainly in the second phase of fattening. The supplementation of cellobiose in LSF groups delayed and reduced progressively mortality, and only the intermediate dose of cellobiose (3.5 g/L) limited the mortality in the second phase of fattening in HSF groups.

From 26 to 39 d of age, rabbits fed HSF diet reduced feed intake (by 6%. $P = 0.002$) and growth rate (by 9%. $P = 0.001$), with no influence on feed efficiency (Table 6). A trend for compensatory growth rate was observed in rabbits fed HSF diet from 39 to 56 d of age ($P = 0.088$), and no effect of the level of soluble fibre was detected on the growth rate in the whole experimental period.

The level of cellobiose also influenced some growth traits alone or in combination with the level of soluble fibre. The increase of the level of cellobiose tended to reduce the feed intake from 26 to 39 d of age ($P = 0.077$), but did not influenced directly the growth rate in this period. In fact, growth rate evolved quadratically in LSF groups, showing a maximal value for rabbits from LSF3.5 group ($P < 0.001$), that led to a similar evolution of feed efficiency, being also maximal for rabbits of this group ($P = 0.006$). The cellobiose intake estimated for this period was equivalent to its inclusion in the diet at a 0.5 and 1.0% for LSF3.5, LSF7 groups, respectively, and 0.7 and 1.4% for HSF3.5 and HSF7 groups, respectively. In the second phase of fattening (39-56 d), once the experimental diets

Table 6. Effect of the level of cellobiose in drinking water (from 26 to 42 d of age) and dietary soluble fibre (from 26 to 39 d of age) on the growth performance of rabbits. (*Experiment 2*)

Soluble fibre	Low (LSF)			High (HSF)			SEM	Cov ²	P- value				
Cellobiose, g/L	0.00	3.50	7.00	0.00	3.50	7.00			Soluble fibre	Cellobiose		Soluble fibre × Cellobiose	
n ¹	22	26	29	24	27	24				Lineal	Quadratic	Lineal	Quadratic
26-39 d ³													
Live weight 26 d, g	464	457	452	452	442	446	13.2	-	0.15	0.33	0.64	0.76	0.68
Feed intake, g/d	93.8	93.0	87.6	87.4	84.4	85.5	2.31	<0.001	0.002	0.077	0.94	0.33	0.26
Weight gain, g/d	45.7	51.2	45.8	43.9	43.1	45.1	1.35	<0.001	0.001	0.60	0.059	0.67	0.002
G:F, g/g	0.489	0.553	0.522	0.516	0.513	0.525	0.0139	0.38	0.78	0.14	0.088	0.39	0.022
Water intake, g/d ⁴	135	134	126	182	176	178	4.22	-	-	-	-	-	-
Cellobiose intake, g/d	0.00	0.467	0.882	0.00	0.616	1.24	0.015	-	-	-	-	-	-
Mortality, %	25.0	6.68	3.13	6.26	3.34	6.26	-	-	0.41	0.11	0.47	0.11	0.84
39-56 d ⁵													
Live weight 39 d, g	1047	1119	1049	1024	1014	1040	17.5	<0.001	0.001	0.60	0.059	0.67	0.002
Feed intake, g/d	162	155	148	172	154	155	6.31	0.094	0.31	0.019	0.36	0.85	0.34
Weight gain, g/d	49.8	50.8	51.3	54.2	49.1	53.1	1.07	0.042	0.088	0.87	0.021	0.24	0.011
G:F, g/g	0.320	0.329	0.348	0.344	.0320	.0342	0.0113	0.38	0.71	0.28	0.15	0.21	0.37
Mortality, %	6.26	6.68	6.26	18.8	6.68	18.8	-	-	0.11	1.00	0.34	1.00	0.30
26-56 d													
Live weight 56 d, g	1896	1982	1919	1946	1848	1945	26.1	<0.001	0.34	0.66	0.60	0.62	<0.001
Feed intake, g/d	132	128	122	135	124	125	4.16	0.013	0.88	0.012	0.43	0.95	0.27
Weight gain, g/d	48.1	50.9	48.9	49.7	46.5	49.7	0.87	<0.001	0.34	0.66	0.60	0.62	<0.001
G:F, g/g	0.372	0.399	0.402	0.388	0.377	0.397	0.0092	0.34	0.62	0.041	0.83	0.26	0.076
Mortality, %	31.3	13.3	9.38	25.0	10.0	25.0	-	-	0.66	0.098	0.11	0.098	0.42

¹ n = number of rabbits at the end of the fattening period and used to calculate growth traits. For mortality the initial number of rabbits was 32/treatment, except for treatments LSF3.5 and HSF3.5 where n = 30. ² Live weight at weaning as covariate. ³ Period where the rabbits were fed with the 3 cellobiose concentrations in drinking water, in combination of 2 levels of soluble fibre (low:LSF 71.9 g/kg DM; high:HSF 130 g/kg DM). ⁴ Estimated according to Delgado et al. (2015) that obtained a ratio water to feed intake of 1.44 and 2.08 for LSF and HSF diets, respectively. ⁵ Period where the rabbits received a standard feed and continued with additive in water until 42d.

Figure 3. Effect of the level of cellobiose in drinking water and dietary soluble fibre in the diet (low 71.9 and high 130 g/kg DM of soluble fibre, respectively) on the accumulated mortality along the fattening period of rabbits weaned at 26 d of age. The vertical discontinuous line at 39 d indicates the change to a common commercial diet and the vertical continuous line at 42 d of age indicates the removal of the cellobiose supplementation. (*Experiment 2*)

were replaced by a standard diet and cellobiose was no longer supplemented from 42 d of age on, feed intake linearly decreased in the groups that had drunk water with increasing doses of cellobiose ($P = 0.019$). In this period, for growth rate, a quadratic effect of cellobiose supplementation was observed in HSF fed rabbits, showing those of HSF3.5 group the minimal value ($P < 0.001$), with no effect on LSF group. No changes were observed in this period on the feed efficiency. In the whole experimental period, the inclusion of cellobiose reduced linearly feed intake and improved feed efficiency ($P \leq 0.041$), while growth rate evolved with cellobiose supplementation quadratically but in a different way depending on the level of soluble fibre. Rabbits fed LSF3.5 showed a maximal weight gain whereas those fed HSF3.5 a minimal value ($P \leq 0.017$).

The increase of soluble fibre tended to increase the relative weight of the digestive tract and the stomach ($P \leq 0.091$), with no influence on the caecum relative weight (Table 7). When no cellobiose was supplemented the increase of dietary soluble fibre tended to reduce the relative liver weight ($P = 0.061$). The LSF3.5 group tended to reduce the relative liver weight ($P = 0.064$) compared to LSF0 and LSF7, with no effect on HSF groups. The specific jejunum sucrose activity at 39 d of age was not affected by any treatment.

4.1.3. Experiment 3: “Effect of cellobiose supplementation on growth performance in rabbits fed a low soluble fibre diet”.

The increase of cellobiose concentration from 26 to 40 d of age tended to decrease both the growth rate and feed efficiency ($P \leq 0.098$) with no influence on feed intake or mortality (Table 8, Figure 4). From 40 to 50 d of age on, the increase of the cellobiose level had no influence on feed intake or growth rate but it linearly improved the feed efficiency ($P = 0.009$). In these periods, the estimated cellobiose intake was equivalent to its inclusion in the diet at a 0.5 and 1.0% concentration for 3.5 and 7.0 groups, respectively. No effect of the cellobiose level was found from 50 to 61 d of age or in the whole experimental period, but a trend to linearly reduce mortality ($P = 0.092$, Figure 4). The mortality in the group with no cellobiose supplementation occurred mostly two weeks after weaning with a minor rise at the

Table 7. Effect of the level of cellobiose in drinking water and dietary soluble fibre on the digestive organs, and jejunal sucrose activity in 39 d of age rabbits. (*Experiment 2*)

Soluble fibre	Low (LSF)			High (HSF)			SEM	<i>P- value</i>				
Cellobiose, g/L	0.0	3.5	7.0	0.0	3.5	7.0		Soluble fibre	Cellobiose		Soluble fibre × Cellobiose	
									Lineal	Quadratic	Lineal	Quadratic
Body weight, g	1077	1053	1110	1076	1057	1077	56.9	0.75	0.65	0.36	0.68	0.76
Relative weight, % BW												
Digestive tract ¹	25.6	25.9	25.8	28.4	25.9	28.4	1.24	0.081	0.92	0.31	0.94	0.22
Stomach ²	6.45	6.64	6.65	7.20	6.61	7.69	0.41	0.091	0.41	0.30	0.73	0.21
Caecum ³	9.39	10.2	10.8	10.6	10.0	10.6	0.68	0.59	0.30	0.68	0.29	0.58
Liver ⁴	5.96	4.89	5.38	5.05	5.32	5.20	0.33	0.43	0.52	0.32	0.27	0.099
Sucrose activity												
Sucrose activity, μmol glucose/g protein	248	321	274	210	243	267	96.8	0.15	0.23	0.28	0.66	0.35
Protein, %	4.08	4.03	3.78	3.91	3.81	3.99	0.64	0.77	0.62	0.92	0.36	0.55

¹ This includes all the rabbit organs after they are removing from the body. ² This make reference to the stomach weight relate to the body weight when is full of content. ³ This make reference to the caecum weight relate to the body weight when is full of content. ⁴ This makes reference to the liver weight relate to the body weight. Low and high soluble fibre content in the diet (71.9 and 130 g/kg DM, respectively).

Table 8. Effect of the level of cellobiose in drinking water (from 26 to 50 d of age) on traits in rabbits. (*Experiment 3*)

n ¹	Cellobiose, g/L			SEM	Cov ²	P-value	
	0.0	3.5	7.0			Lineal	Quadratic
	48	52	55				
26-40 d³							
Live weight 26 d, g	452	445	442	11.5	-	0.33	0.85
Feed intake, g/d	78.1	78.3	77.1	1.36	<0.001	0.60	0.69
Weight gain, g/d	50.4	49.5	47.9	0.98	<0.001	0.055	0.76
G:F, g/g	0.644	0.637	0.623	0.0100	0.56	0.098	0.79
Water intake, g/d ⁴	115	115	113	2.00	-	-	-
Cellobiose intake, g/d	0.00	0.401	0.791	0.010	-	-	-
Mortality, %	9.77	3.32	8.16	-	-	0.75	0.14
40-50 d⁵							
Live weight 40 d, g	1150	1138	1116	13.7	<0.001	0.055	0.75
Feed intake, g/d	118	118	116	3.16	0.12	0.63	0.82
Weight gain, g/d	43.1	47.2	46.3	1.51	0.17	0.14	0.17
G:F, g/g	0.349	0.397	0.405	0.0150	0.003	0.009	0.26
Water intake, g/d ⁶	169	169	166	4.51	-	-	-
Cellobiose intake, g/d	0.00	0.592	1.16	0.016	-	-	-
Mortality, %	9.77	9.77	3.32	-	-	0.14	0.34
50-61 d							
Live weight 50 d, g	1583	1610	1579	22.2	<0.001	0.88	0.25
Feed intake, g/d	135	137	135	3.92	0.012	0.95	0.62
Weight gain, g/d	44.7	46.6	47.5	2.10	0.20	0.35	0.83
G:F, g/g	0.308	0.352	0.347	0.0196	0.85	0.15	0.29
Mortality, %	3.32	3.32	0.10	-	-	0.12	0.25
26-61 d							
Live weight 61 d, g	2074	2124	2103	29.1	<0.001	0.48	0.33
Feed intake, g/d	107	108	106	2.02	<0.001	0.73	0.61
Weight gain, g/d	46.5	48.0	47.4	0.83	0.001	0.48	0.32
G:F, g/g	0.435	0.447	0.446	0.0062	0.065	0.21	0.35
Mortality, %	22.7	16.2	11.4	-	-	0.092	1.00

¹ n= number of rabbits at the end of the fattening period and used to calculate growth traits. For mortality the initial number of rabbits was 62/treatment. ² Live weight at weaning as covariate. ³ Period where the rabbits were supplied the 3 concentrations of cellobiose in drinking water in combination a low soluble fibre diet (71.9 g/kg DM). ⁴ Estimated according to Delgado et al. (2015), that obtained a ratio water to feed intake of 1.47. ⁵ Period where the rabbits received a standard feed and continued with cellobiose supplementation in water until 50 d. ⁶ Estimated according to Delgado et al. (2015) that obtained a ratio water to feed intake of 1.43.

Figure 4. Effect of the level of cellobiose in drinking water on the accumulated mortality along the fattening period of rabbits weaned at 26 d of age. The vertical discontinuous line at 40 d indicates the change to a commercial diet and the vertical continuous line at 50 d of age indicates the suspension of the cellobiose treatment. Soluble fibre content in the experimental diet (71.9 g/kg DM). (*Experiment 3*)

end of fattening (Figure 4). The supplementation of cellobiose delayed and reduced progressively mortality, but did not reduce it below the threshold of 10%.

4.1.4. Experiment 4: “Effect of pre- and post-weaning cellobiose supplementation on growth performance and health in rabbits”.

The supplementation of cellobiose in the pre-weaning period reduced feed intake (by 7%. $P = 0.042$) with no influence on growth rate, feed efficiency, or milk intake (27.3 ± 7.0 g of milk/kit, data not shown). Just after weaning (29-43 d of age), rabbits supplemented with cellobiose increased weight gain and feed efficiency (by 20%. $P < 0.001$), with no effect on feed intake (Table 9). From 43 to 57 d of age those groups supplied with no cellobiose after weaning improved weight gain and feed efficiency (by 9%. $P = 0.003$), especially the group that never received cellobiose. In the whole experimental period, the groups supplemented with cellobiose after weaning tended to show a higher growth rate ($P = 0.056$) and those supplied with cellobiose before weaning tended to have a higher mortality ($P = 0.10$. Figure 5), mainly due to the group that received cellobiose throughout the experiment. Mortality in the group supplemented with cellobiose throughout the experimental period was very high just after weaning, even when the cellobiose supplementation was suspended at 50 d of age.

Rabbits supplemented with cellobiose after weaning had a lighter liver than those that drank only water (by 9%. $P = 0.047$). The cellobiose supplementation did not influence the weight of the digestive tract or the caecal pH (Table 10).

Table 9 Effect of period and dose of cellobiose supplementation (pre-weaning vs. post-weaning, and 0 vs. 7 g/L) on growth traits of growing rabbits from 22 to 57 d of age. (*Experiment 4*)

Pre-weaning treatment	Water (w)		Cellobiose (c)		SEM	Cov ²	P-value			
	Water (W)	Cellobiose (C)	Water (W)	Cellobiose (C)			Pre-weaning	Post-weaning		
							w vs. c	wW + wC vs. cW + cC	wW + cW vs. wC + cC	wW + cC vs. cW + wC
Post-weaning treatment	Water (W)	Cellobiose (C)	Water (W)	Cellobiose (C)						
n¹	20		20							
22-29 d³										
Live weight at 22 d, g/kit	354		353		6.18	-	0.50	-		-
Feed intake, g/kit and d	11.8		11.0		0.331	0.41	0.042	-		-
Weight gain, g/d and kit	18.4		18.0		1.02	0.058	0.79	-		-
G:F, g/g	0.617		0.634		0.035	0.87	0.74	-		-
Water intake, g/d and kit ⁴	15.9		14.8		0.447	-	-	-		-
Cellobiose intake, g/d and kit	0.00		0.104		0.0027	-	-	-		-
n²	36	36	35	30						
29-43 d⁵										
Live weight at 29 d, g	524	517	525	528	12.8	-	-	0.61	0.88	0.70
Feed intake, g/d	110	115	112	112	2.73	0.063	-	0.95	0.37	0.34
Weight gain, g/d	42.5	52.1	42.3	51.3	1.64	0.35	-	0.74	<0.001	0.82
G:F, g/g	0.391	0.453	0.379	0.460	0.0125	0.087	-	0.83	<0.001	0.40
Water intake, g/d ⁶	163	170	166	166	4.05	-	-	-	-	-
Cellobiose intake, g/d	0.00	1.19	0.00	1.17	0.020	-	-	-	-	-
Mortality, %	2.57	7.51	5.01	12.5	0.703	-	-	0.37	0.12	0.93
43-57 d⁷										
Live weight at 43 d, g	1119	1253	1116	1242	22.9	<0.001	-	0.74	<0.001	0.82
Feed intake, g/d	186	184	182	183	6.54	0.49	-	0.65	0.88	0.84
Weight gain, g/d	61.0	53.8	55.5	52.8	1.64	0.33	-	0.050	0.003	0.18
G:F, g/g	0.336	0.295	0.306	0.294	0.0096	0.69	-	0.066	0.003	0.087
Mortality, %	7.70	2.52	7.51	12.5	-	-	-	0.21	0.66	0.19
29-57 d										
Live weight at 57 d, g	1971	2009	1891	1982	33.3	<0.001	-	0.11	0.056	0.43
Feed intake, g/d	148	150	147	148	4.23	0.26	-	0.71	0.87	0.88
Weight gain, g/d	51.7	53.1	48.9	52.1	1.19	0.98	-	0.11	0.056	0.43
G:F, g/g	0.357	0.357	0.335	0.358	0.0090	0.29	-	0.20	0.15	0.15
Mortality, %	10.3	10.0	12.5	25.0	0.466	-	-	0.10	0.52	0.48

¹ n = number of litters used during pre-weaning period (5 kits/litter and treatment). ² n = number of rabbits at the end of the fattening period (post-weaning) and used to calculate growth traits. For mortality the initial number of rabbits was 40/treatment. ³ Period where the rabbits were fed with 2 concentrations of cellobiose in drinking water in combination of a standard commercial diet, but still lactating. ⁴ Estimated according to Delgado et al. (2015), that obtained a ratio water to feed intake of 1.35. ⁵ Period where the rabbits were fed with 2 concentrations of cellobiose in drinking water in combination of a standard commercial diet. ⁶ Estimated according to Delgado et al. (2015) that obtained a ratio water to feed intake of 1.48. ⁷ Cellobiose supplementation was suspended at 50 d of age.

Table 10. Effect of cellobiose supplementation on digestive traits in 39 days old rabbits. (*Experiment 4*)

Pre-weaning treatment	Water (w)		Cellobiose (c)		SEM ¹	P-value		
Post-weaning treatment	Water (W)	Cellobiose (C)	Water (W)	Cellobiose (C)		wW + wC vs. cW + cC	wW + cW vs. wC + cC	wW + cC vs. cW + wC
Body weight at 39 d, g	1053	1046	1047	976	43.6	0.39	0.38	0.48
Relative weight, % BW								
Digestive tract	25.5	26.8	24.9	25.9	0.974	0.39	0.22	0.87
Liver	6.06	5.41	6.12	5.61	0.270	0.64	0.047	0.80
Caecal pH	5.80	5.72	5.80	5.71	0.088	0.81	0.46	0.86

¹n = 10.

Figure 5. Effect of the level of cellobiose in drinking water on the accumulated mortality along the post-weaning period in rabbits weaned at 29 d of age and assigned to treatments (0 or 7 g cellobiose/L at 22 d of age. No mortality was recorded before weaning). The vertical continuous line at 50 d of age indicates the suspension of the cellobiose treatment. (*Experiment 4*)

4.2.OBJECTIVE 2:

Evaluate the influence of cellobiose and soluble fibre supplementation on the *in vitro* caecal fermentation of cellobiose and other carbohydrates-rich feedstuffs.

4.2.1. Experiment 5: “*In vitro* caecal fermentation of carbohydrate-rich feedstuffs in rabbits as affected by substrate pre-digestion and donors’ diet”.

The solubility of substrates during the pre-digestion was that expected, but PEC flocculated under the acidic conditions of the first step of the pre-digestion. Table 11 shows the effects of pre-digestion and donors’ diet on gas production kinetics and VFA production in the cultures without substrate (blanks). Pre-digested blanks had greater ($P \leq 0.015$) values of Vf (about three fold increase), μ_m and Ti, total VFA production (six fold increase) and butyrate and isovalerate proportions, as well as lower ($P \leq 0.004$) k values and acetate proportions, than those untreated. Donors’ diet affected k and Ti values, with the LSF-inoculated blanks having greater k ($P = 0.017$) and lower Ti values ($P = 0.006$) than those HSF-inoculated. There were no pre-digestion $\times$ donors’ diet interactions ($P > 0.05$) for any gas parameter in the blanks with the exception of total VFA production and isovalerate molar proportion ($P \leq 0.010$), and a trend ($P \leq 0.092$) was detected for Vf and acetate proportion.

Multiple interactions among the studied factors (pre-digestion, donors’ diet and substrate) were observed for gas production parameters in the cultures with substrate (Table 12, Figure 6, and Annex 1). Pre-digestion $\times$ substrate and donors’ diet $\times$ substrate interactions were detected for Vf, lag time and μ_m values ($P \leq 0.014$). As shown in Figure 7, for CEL substrate Vf values were greater ($P \leq 0.033$) and lag time values were lower ($P \leq 0.039$) in the pre-digested cultures than in those untreated (333 vs. 256 ml/g DM, and 20.5 vs. 29.9 h, respectively), and in the LSF-inoculated cultures compared with those HSF-inoculated (318 vs. 271 ml/g DM, and 20.3 vs. 30.1 h, respectively). In contrast, Vf values were unaffected ($P \geq 0.174$) by any studied factor for the other substrates.

Similarly to that observed for CEL, the pre-digestion shortened lag time values for SBP (13.6 vs. 19.7 h; $P = 0.042$), but enlarged it for PEC (19.8 vs. 8.6 h;

Table 11. Effect of pre-digestion and type of diet fed to donors' rabbits of soft faeces (low soluble fibre [LSF] or high soluble fibre [HSF]) on gas production kinetics and volatile fatty acid (VFA) production and molar proportions in blanks (cultures without substrate) (n = 4 rabbits/diet)¹ (*Experiment 5*)

Pre-digestion	No		Yes		SEM ²		P-value		
Donors' diet	LSF	HSF	LSF	HSF	Pre-digestion/ donors' diet ²	Pre-digestion × donors' die	Pre-digestion	Donors' diet	Pre-digestion × donors' diet
Gas production kinetics³									
V _f , ml/culture	4.63	4.26	13.8	15.7	0.435	0.615	<0.001	0.240	0.092
k, %/h	0.179	0.127	0.098	0.082	0.0086	0.0122	<0.001	0.017	0.159
Lag time, h	0.000	0.272	0.000	0.000	0.0963	0.1363	0.337	0.337	0.337
μ _m , ml/h	0.861	0.539	1.35	1.28	0.0893	0.1264	<0.001	0.148	0.334
T _i , h	2.91	4.26	5.25	6.15	0.237	0.335	<0.001	0.006	0.512
VFA production									
Total VFA (mmol/l)	1.13	0.757	6.53	5.31	0.0976	0.1380	<0.001	<0.001	0.010
Molar VFA proportions (mol/100 mol)									
Acetate	100	100	91.1	97.5	1.13	1.60	0.004	0.069	0.069
Propionate	0.00	0.00	1.05	0.66	0.0439	0.621	0.193	0.760	0.760
Butyrate	0.00	0.00	3.39	0.99	0.224	0.775	0.015	0.148	0.148
Isobutyrate	0.00	0.00	0.64	0.32	0.549	0.317	0.337	0.337	0.337
Isovalerate	0.00	0.00	3.82	0.86	0.313	0.443	<0.001	0.006	0.006

¹ *In vitro* incubations for determining gas production parameters and VFA production lasted for 144 and 24 h, respectively. ² SEM of the mean values for pre-digestion and donors' diet were the same for all variables. ³ V_f: asymptotic gas production; k: fractional rate of gas production; Lag time: initial delay in the onset of gas production; μ_m: maximum gas production rate; T_i: time when μ_m is reached.

Table 12. Effect of pre-digestion, type of diet fed to donors' rabbits of soft faeces (low soluble fibre [LSF] or high soluble fibre [HSF]) and incubated substrate on gas production kinetics in 144 h *in vitro* incubations (n = 4 rabbits/diet)¹ (*Experiment 5*)

	Vf (ml/g DM)	k (%/h)	Lag time (h)	μ_m (ml/h)	T _i (h)
Pre-digestion					
No	170	0.032	15.8	5.96	37.2
Yes	198	0.042	13.1	9.79	30.4
SEM	4.68	0.0030	0.98	0.771	1.37
Donors' diet					
Low soluble fibre	187	0.042	13.6	9.58	31.9
High soluble fibre	180	0.032	15.3	6.17	35.7
SEM	4.68	0.0030	0.98	0.771	1.37
Substrate					
Cellobiose	295 ^d	0.069 ^b	25.3 ^d	21.0 ^c	34.4 ^b
Pectin	261 ^c	0.024 ^a	11.3 ^b	6.25 ^{ab}	36.3 ^b
Sugar beet pulp	142 ^b	0.021 ^a	16.7 ^c	3.05 ^a	41.7 ^c
Wheat straw	36.8 ^a	0.035 ^a	4.52 ^a	1.20 ^a	22.7 ^a
SEM	6.62	0.0042	1.388	1.089	1.94
P-value²					
Pre-digestion	<0.001	0.029	0.065	0.001	0.001
Donors' diet	0.292	0.028	0.214	0.003	0.056
Substrate	<0.001	<0.001	<0.001	<0.001	<0.001
Pre-digestion × donors' diet	0.886	0.132	0.786	0.017	0.601
Pre-digestion × substrate	0.001	0.517	0.003	0.003	0.535
Donors' diet × substrate	0.009	0.006	0.014	<0.001	0.021
Pre-digestion × donors' diet × substrate	0.116	0.006	0.471	<0.001	0.432

^{a-d} For each item, means for substrates in the same column with different letters differ significantly (P<0.05). ¹ V_f: asymptotic gas production; k: fractional rate of gas production; Lag time: initial delay in the onset of gas production; μ_m : maximum gas production rate; T_i: time when μ_m is reached. ² Interactions are shown in Figure 7.

Figure 6. Effect of pre-digestion, type of diet fed to donors' rabbits of soft faeces (low soluble fibre [LSF] 84.0 g/kg DM or high soluble fibre [HSF] 130 g/kg DM) and incubated substrate on gas production kinetics in 144 h of *in vitro* incubations (n = 4 rabbits/diet. Standard error = 9.7 ml/g DM substrate). (*Experiment 5*)

Figure 7. Effect of pre-digestion (Yes vs. No), type of diet fed to donors' rabbits of soft faeces (low soluble fibre [LSF ■] 84.0 g/kg DM or high soluble fibre [HSF ▨] 130 g/kg DM) and incubated substrate (D-cellobiose (CEL), sugar beet pulp pectin (PEC), sugar beet pulp (SBP) and wheat straw (WS)) on gas production kinetics in 144 h *in vitro* incubations (n = 4 rabbits/diet). A) V_f : asymptotic gas production; B) k : fractional rate of gas production; C) L (lag time): initial delay in the onset of gas production; D) μ_m : maximum gas production rate; E) T_i : time when μ_m is reached. a, b: Within each substrate, columns with different letters differ ($P < 0.05$). Bars indicate the standard error. Within each substrate, * and † indicate an effect ($P < 0.05$) of pre-digestion and donors' diet, respectively. (Experiment 5)

$P = 0.044$) and had no effect for WS (4.4 vs. 4.7 h; $P = 0.804$). No influence ($P \geq 0.136$) of donors' diet on lag time values was observed for SBP, PEC and WS. The LSF-inoculum decreased T_i values for CEL (27.8 vs. 40.9 h for LSF and HSF inoculum, respectively; $P = 0.021$), but had no effect for the other substrates, thus resulting in a donors' diet $\times$ substrate interaction for this parameter. Pre-digestion $\times$ donors' diet $\times$ substrate interactions were detected for k and μ_m ($P \leq 0.006$). These triple interactions were explained by the increase of k and μ_m values when the LSF-inoculum was used to ferment the pre-digested CEL compared with the HSF-inoculum (0.111 vs. 0.042 %/h, and 40.8 vs. 12.2 ml/h, respectively), whereas no effect was detected for SBP and WS substrates. For PEC, the pre-digestion increased k and μ_m values only with the HSF-inoculum (0.035 vs. 0.013 %/h and 10.1 vs. 3.3 ml/h, respectively).

Pre-digestion $\times$ substrate and donors' diet $\times$ substrate interactions were also observed for total VFA production ($P \leq 0.049$; Table 13 and Figure 8). Whereas the pre-digestion increased total VFA production for CEL (13.0 vs. 6.1 mmol/l; $P = 0.036$), the opposite was observed for WS (6.6 vs. 8.2 mmol/l; $P = 0.022$), and no effect was detected for SBP and WS ($P \geq 0.502$). Furthermore, the LSF-inoculum increased total VFA production for CEL (14.4 vs. 5.4 mmol/l. $P = 0.036$), but decreased it for WS (6.3 vs. 8.5 mmol/l; $P = 0.022$). No effects of either the pre-digestion or the donors' diet on total VFA production were observed for PEC and SBP.

A pre-digestion $\times$ donors' diet $\times$ substrate interaction ($P \leq 0.001$) was observed for molar proportions of acetate and butyrate. The pre-digestion of CEL caused a sharply decrease in the proportion of acetate and an increase in that of butyrate (by 37 and 36 percentage units, respectively) when it was fermented with the LSF-inoculum, but these effects were not observed with the HSF-inoculum. In contrast, similar qualitative changes in VFA profile (decrease in acetate and increase in butyrate by 9 and 4 percentage units, respectively) were observed by pre-digesting WS with the HSF-inoculum. For SBP, pre-digestion caused similar changes in VFA profile with both inocula. Finally, the pre-digestion of all substrates resulted in greater ($P \leq 0.006$) proportions of propionate, isobutyrate and isovalerate than untreated ones. No valerate was detected in any sample.

Table 13. Effect of pre-digestion, type of diet fed to donors' rabbits of soft faeces (low soluble fibre [LSF] or high soluble fibre [HSF]) and incubated substrate on gas production, total volatile fatty acid (VFA) production and individual VFA profile after 24 h of *in vitro* fermentation (n = 4 rabbits/diet). (*Experiment 5*)

	Gas production (ml/g DM)	Total VFA (mmol/l)	Molar proportions (mol/100 mol)				
			Acetate	Propionate	Butyrate	Isobutyrate	Isovalerate
Pre-digestion							
Yes	77.7	10.2	87.1	2.78	8.32	0.66	1.18
No	30.5	8.99	98.8	0.00	1.17	0.00	0.00
SEM	5.273	0.854	1.17	0.408	0.964	0.160	0.213
Donors' diet							
LSF	69.6	11.1	92.4	0.86	6.14	0.25	0.32
HSF	38.5	8.03	93.5	1.93	3.35	0.40	0.87
SEM	5.273	0.854	1.17	0.408	0.964	0.160	0.213
Substrate							
Cellobiose	94.4 ^b	9.61 ^a	85.0 ^a	0.47	13.5 ^b	0.36	0.67
Pectin	78.4 ^b	14.2 ^b	96.1 ^b	1.69	1.36 ^a	0.31	0.50
Sugar beet pulp	28.5 ^a	7.14 ^a	96.7 ^b	1.11	1.55 ^a	0.20	0.48
Wheat straw	15.0 ^a	7.40 ^a	94.0 ^b	2.30	2.58 ^a	0.45	0.72
SEM	7.457	1.207	1.65	0.578	1.363	0.226	0.301
<i>P</i>-value¹							
Pre-digestion	<0.001	0.338	<0.001	<0.001	<0.001	0.006	<0.001
Donors` diet	<0.001	0.013	0.541	0.070	0.046	0.510	0.073
Substrate	<0.001	<0.001	<0.001	0.151	<0.001	0.887	0.925
Pre-digestion × donors' diet	0.006	0.638	0.193	0.070	0.006	0.510	0.073
Pre-digestion × substrate	<0.001	0.049	0.003	0.151	<0.001	0.887	0.925
Donors' diet × substrate	<0.001	0.010	0.005	0.583	0.001	0.780	0.419
Pre-digestion × donors' diet × substrate	<0.001	0.327	0.001	0.583	<0.001	0.780	0.419

^{a-b} For each item, means for substrates in the same column with different letters differ significantly (P<0.05). ¹ Interactions are shown in Figure 8.

Figure 8. Effect of pre-digestion (Yes vs. No), type of diet fed to donors' rabbits of soft faeces (low soluble fibre [LSF ■] 84.0 g/kg DM or high soluble fibre [HSF ▨] 130 g/kg DM) and incubated substrate (D-cellobiose (CEL), sugar beet pulp pectin (PEC), sugar beet pulp (SBP) and wheat straw (WS)) on total volatile fatty (VFA) production and molar proportions of individual VFA after 24 h of *in vitro* incubation (n = 4 rabbits/diet). a, b: Within each substrate, columns with different letters differ (P < 0.05). Bars indicate the standard error. Within each substrate, * and † indicate an effect (P < 0.05) of pre-digestion and donors' diet, respectively. (Experiment 5)

4.2.2. Experiment 6: “Effect of cellobiose supplementation and dietary soluble fibre content on *in vitro* caecal fermentation of carbohydrate-rich substrates in rabbits”.

Table 14 shows the influence of cellobiose supplementation and donors’ diet on gas production parameters and VFA production in the cultures without substrate (blanks). There were no cellobiose $\times$ donors’ diet interactions with the exception of a trend for Vf ($P = 0.086$) and isovalerate proportion ($P = 0.054$). Values of Vf, k and T_i were quadratically influenced by cellobiose supplementation ($P = 0.003$, 0.002 and 0.001 , respectively), with blanks from rabbits receiving the 7.5 dose of cellobiose showing lower K values ($P < 0.05$) and greater T_i values ($P < 0.05$) than those from rabbits receiving the 0 and 15 doses (0.090, 0.072 and 0.059 %/h for 0, 7.5 and 15 doses, respectively, and 5.70, 6.99 and 5.55 h, respectively; values averaged across diets). There were no effects of donors’ diet on any gas production parameter ($P = 0.31$ to 1.00).

Total VFA production in the blanks increased linearly ($P = 0.001$) by cellobiose supplementation, with no differences between the 7.5 and 15 doses (5.92, 9.28 and 9.71 mmol/L for 0, 7.5 and 15 doses, respectively; values averaged across diets). In contrast, cellobiose supplementation did not affect VFA profile ($P > 0.05$), with the exception of the molar proportion of butyrate ($P = 0.036$), which was greater ($P < 0.05$) for the 7.5 dose than for the 0 dose with both donors’ diets. Total VFA production was greater for LSF compared with the HSF inoculum (9.11 vs. 7.49 mmol/L; $P = 0.035$), but donors’ diet had no influence on VFA profile in the blanks ($P = 0.18$ to 0.78).

Table 15 and Figures 9 and 10 show the influence of cellobiose supplementation and donors’ diet on gas production kinetics of the incubated substrates. As expected, gas production parameters differed widely ($P < 0.001$) among substrates. There were also marked differences among substrates in the response to experimental factors, which lead to cellobiose $\times$ donors’ diet $\times$ substrate interactions ($P \leq 0.003$) for K, *Lag* and μ_m . As shown in Figure 9 and Annex 2, gas production kinetics of SBP and WS was not modified either by cellobiose supplementation or donor’s diet ($P \geq 0.12$), but some effects were observed for the rest of substrates, being those more pronounced for CEL than for GLU and PEC.

Table 14. Effect of cellobiose supplementation and diet fed to donors' rabbits of soft faeces on gas production kinetics, total volatile fatty acid (VFA) production and VFA profile in cultures without substrate (blanks; n = 4)¹. (*Experiment 6*)

Donors' diet	Low soluble fibre			High soluble fibre				P-value		
Cellobiose, (g/L)	0.0	7.5	15.0	0.0	7.5	15.0	SEM	Cellobiose ³	Donors' diet	Cellobiose × donors' diet
Gas production kinetics ³										
Vf, ml	13.8 ^{AB}	16.1 ^B	13.6 ^A	15.7 ^{AB}	17.1 ^B	10.6 ^A	1.049	0.002	0.99	0.086
k, %/h	0.098 ^B	0.072 ^A	0.092 ^B	0.082 ^B	0.071 ^A	0.097 ^B	0.0065	0.007	0.46	0.29
Lag, h	0.00	0.00	0.00	0.00	0.00	0.00	0.000	1.00	1.00	1.00
μ _m , ml/h	1.35	1.17	1.21	1.28	1.22	1.01	0.083	0.068	0.31	0.36
T _i , h	5.24 ^A	6.93 ^B	5.62 ^A	6.15 ^A	7.05 ^B	5.27 ^A	0.410	0.003	0.51	0.33
VFA production										
Total VFA (mmol/L)	6.53 ^A	10.4 ^B	10.4 ^B	5.31 ^A	8.15 ^B	9.01 ^B	0.863	0.001	0.035	0.82
Individual VFA, mol/100 mol										
Acetate	91.1	89.2	87.2	97.5	89.2	89.6	2.661	0.081	0.20	0.47
Propionate	1.05	2.61	4.71	0.66	1.48	1.56	1.365	0.28	0.18	0.60
Butyrate	3.39 ^A	4.72 ^B	4.04 ^{AB}	0.99 ^A	5.19 ^B	5.13 ^B	1.069	0.036	0.75	0.24
Isobutyrate	0.63	0.52	1.16	0.00	0.00	0.81	0.518	0.34	0.25	0.97
Isovalerate	3.82	2.92	2.86	0.86	4.15	2.95	0.840	0.37	0.44	0.054

^{A-B} For cellobiose doses, means in the same row with different capital letters differ ($P < 0.05$). ¹ *In vitro* incubations for determining gas production parameters lasted for 144 h and VFA production was measured at 24 h. ² P values for linear and quadratic effects of cellobiose supplementation were 0.021 and 0.003 for Vf, 0.51 and 0.002 for k, 0.55 and 0.001 for T_i, 0.001 and 0.066 for total VFA and 0.041 and 0.11 for butyrate proportion, respectively. ³ V_f: asymptotic gas production; k: fractional rate of gas production; Lag: initial delay in the onset of gas production; μ_m: maximum gas production rate; T_i: time when μ_m is reached.

Table 15. Effects of cellobiose supplementation and diet fed to donors' rabbits of soft faeces on gas production kinetics of different substrates in 144 h *in vitro* incubations (n=4). (*Experiment 6*)

	Gas production parameters ¹				
	V _f , ml/g DM	K, %/h	Lag, h	μ _m , ml/h	T _i , h
Cellobiose, g/L					
0.0	220 ^B	0.043	11.8 ^B	10.8	27.9
7.5	214 ^{AB}	0.051	9.13 ^A	12.3	25.3
15.0	201 ^A	0.047	9.83 ^A	11.3	26.0
SEM	4.834	0.0030	0.496	0.836	1.265
Donors' diet					
Low soluble fibre	213	0.052	10.5	13.5	25.9
High soluble fibre	210	0.042	10.0	9.41	26.9
SEM	3.948	0.0025	0.405	0.683	1.033
Substrate					
Cellobiose	323 ^E	0.096 ^C	16.4 ^C	31.7 ^D	23.1 ^{AB}
Glucose	291 ^D	0.045 ^B	7.11 ^A	13.2 ^C	19.4 ^A
Pectin	252 ^C	0.032 ^{AB}	11.6 ^B	8.04 ^B	28.2 ^B
Sugar beet pulp	151 ^B	0.019 ^A	11.1 ^B	2.86 ^A	38.7 ^C
Wheat straw	39.8 ^A	0.044 ^B	5.07 ^A	1.56 ^A	22.6 ^{AB}
SEM	6.245	0.0039	0.641	1.080	1.634
P-value					
Cellobiose ²	0.029	0.18	<0.001	0.41	0.31
Donors' diet	0.63	0.007	0.40	<0.001	0.47
Substrate	<0.001	<0.001	<0.001	<0.001	<0.001
Cellobiose × substrate	0.71	0.12	0.005	0.14	0.31
Donors' diet × substrate	0.002	<0.001	0.50	<0.001	0.59
Cellobiose × donors' diet	0.96	0.012	0.18	0.007	0.42
Cellobiose × donors' diet × substrate	0.81	0.003	0.003	<0.001	0.084

^{A-D} Within each parameter and experimental factor, means in the same column with different letters differ significantly ($P < 0.05$). ¹ V_f: asymptotic gas production; K: fractional rate of gas production; Lag: initial delay in the onset of gas production; μ_m: maximum gas production rate; T_i: time when μ_m is reached. ² P values for linear and quadratic effects of cellobiose supplementation for each substrate are given in Figure 10.

Figure 9. Effect of cellobiose supplementation (0.0, 7.5 and 15 g/L), diet fed to donors' rabbits of soft faces (low or high soluble fibre, 84.0 and 130 g/kg DM, respectively) and incubated substrate on gas production kinetics in 144 h *in vitro* incubations (n = 4; Standard error = 10.1 ml/g DM substrate). (*Experiment 6*)

Figure 10. Effect of cellobiose supplementation, diet fed to donors' rabbits of soft faces (low soluble fibre or high soluble fibre, 84.0 and 130 g/kg DM, respectively) and incubated substrate (glucose [GLU], cellobiose [CEL], sugar beet pulp pectins (Pectins [PEC]), sugar beet pulp [SBP], and wheat straw [WS]) on gas production parameters in 144 h *in vitro* incubations (n = 4 rabbits/treatment). A) V_f : asymptotic gas production; B) k : fractional rate of gas production; C) Lag : initial delay in the onset of gas production; D) μ_m : maximum gas production rate; E) T_i : time when μ_m is reached. Within each diet (low soluble fibre or high soluble fibre) and for each substrate, P_L and P_Q indicate lineal and quadratic effects ($P < 0.05$) of cellobiose, respectively. Bars indicate the standard error. (Experiment 6)

Cellobiose $\times$ substrate interactions were only observed for *Lag* ($P = 0.005$), as cellobiose supplementation decreased linearly *Lag* values for CEL and PEC ($P = 0.003$ and 0.012 , respectively) but had no effect for the rest of substrates ($P > 0.05$; Figure 10C). Donors' diet $\times$ substrate interactions ($P \leq 0.002$) were found for Vf, k and μ_m . The Vf values for CEL substrate were greater with the LSF inoculum than with HSF one, but the opposite was observed for PEC (Figure 10A), and no effects were detected for GLU, SBP and WS. This effect was specially observed in the first 48 h of gas production (Annex 3). Both k and μ_m values for CEL were greater with the HSF compared with LSF inoculum ($P < 0.001$; 0.119 vs. 0.073 %/h, and 41.5 vs. 21.9 h, respectively), but there were no effects of donors' diet ($P \geq 0.20$) on k and μ_m values for the rest of substrates.

As previously shown, CEL was the most affected substrate by the experimental treatments, and cellobiose $\times$ donors' diet interactions were detected for k , *Lag*, μ_m and T_i values of CEL. Whereas k and μ_m were increased linearly and *Lag* and T_i decreased linearly by cellobiose supplementation with the HSF inoculum ($P = 0.025$, 0.013 , 0.001 and < 0.001 , respectively; Figures 10B to 10E), with the LSF inoculum cellobiose supplementation only affected k values ($P = 0.043$) of CEL, which were quadratically increased and had maximal values for the 7.5 dose (Figure 10B). The incubation of CEL with the HSF0 inoculum resulted in greater *Lag* and T_i values compared with the HSF7.5 and HSF15 inocula, without differences ($P > 0.05$) between the 7.5 and 15 doses of cellobiose.

Cellobiose $\times$ donors' diet interactions were only detected for k and *Lag* parameters for GLU, and for *Lag* for PEC. When both substrates were incubated with the HSF inoculum, cellobiose supplementation decreased linearly *Lag* values for GLU ($P = 0.002$) and increased linearly *Lag* values for PEC ($P = 0.003$).

Values of gas and VFA production at 24 h of incubation are shown in Table 16 and Figure 11. Gas production at 24 h for GLU, PEC and SBP substrates was not affected either by the dose of cellobiose or by donors' diet ($P \geq 0.10$), but significant effects of both factors were detected for CEL, thus leading to interactions among the experimental treatments. Cellobiose $\times$ donors' diet interactions for the 24 h gas production were only detected for CEL and WS substrates ($P = 0.002$ and 0.009 , respectively). For CEL, cellobiose supplementation increased linearly gas production at 24 h with the HSF inoculum ($P < 0.001$; 43.0 , 213 and 253 ml/g DM for 0 , 7.5 and

Table 16. Effects of cellobiose supplementation and diet fed to donors' rabbits of soft faeces on gas production, total volatile fatty acid (VFA) production and VFA profile of different substrates after 24 h *in vitro* fermentation. (*Experiment 6*)

	Gas production (ml/g DM)	Total VFA, mmol / L	Molar proportions (mol / 100 mol) ¹				
			Ac	Pr	But	Isobut	Isoval
Cellobiose, g/L							
0.0	110 ^A	9.76 ^A	89.3	2.23 ^A	7.04	0.524	0.948
7.5	135 ^B	13.9 ^B	85.5	5.85 ^B	7.65	0.668	0.331
15.0	123 ^{AB}	11.0 ^A	85.1	5.72 ^B	8.54	0.114	0.546
SEM	5.84	0.801	1.574	0.987	0.920	0.174	0.199
Donors` diet							
Low soluble fibre	135	12.0	84.1	4.56	10.6	0.338	0.313
High soluble fibre	110	11.0	89.1	4.64	4.85	0.533	0.904
SEM	4.77	0.654	1.286	0.806	0.751	0.142	0.162
Substrate							
Cellobiose	229 ^D	16.8 ^C	68.4 ^A	2.67 ^{AB}	28.1 ^B	0.273 ^{AB}	0.554 ^{AB}
Glucose	212 ^{CD}	6.79 ^{AB}	98.7 ^C	0.00 ^A	1.31 ^A	0.00 ^A	0.00 ^A
Pectin	101 ^B	20.2 ^D	92.5 ^C	5.02 ^{BC}	1.44 ^A	0.514 ^{AB}	0.496 ^{AB}
Sugar beet pulp	42.9 ^A	8.83 ^B	89.8 ^{BC}	6.41 ^{BC}	2.67 ^A	0.436 ^{AB}	0.707 ^{AB}
Wheat staw	28.2 ^A	5.09 ^A	83.7 ^B	8.89 ^C	5.21 ^A	0.954 ^B	1.29 ^B
SEM	7.54	1.035	2.033	1.275	1.188	0.224	0.257
<i>P-value</i>							
Cellobiose ²	0.009	0.001	0.12	0.014	0.53	0.078	0.080
Donors` diet	<0.001	0.29	0.008	0.95	<0.001	0.33	0.012
Substrate	<0.001	<0.001	<0.001	<0.001	<0.001	0.053	0.016
Cellobiose × substrate	<0.001	0.001	0.87	0.56	0.22	0.54	0.91
Donors` diet × substrate	<0.001	0.006	<0.001	0.90	<0.001	0.98	0.57
Cellobiose × donors` diet	0.015	0.76	0.11	0.49	0.005	0.96	0.049
Cellobiose × donors` diet × substrate	<0.001	0.91	0.83	0.91	0.37	0.73	0.24

^{A-B} For each item, means in the same column with different letters differ ($P < 0.05$). ¹ Ac: acetate; Pr: propionate; But: butyrate; Isobut: isobutyrate; Isoval: isovalerate. ² P values for linear and quadratic effects of cellobiose supplementation for each substrate are given in Figure 11.

Figure 11. Effect of cellobiose supplementation, fed to donors' rabbits of soft faces (low soluble fibre or high soluble fibre, 84.0 and 130 g/kg DM, respectively) and incubated substrate (glucose [GLU], cellobiose [CEL], sugar beet pulp pectins (Pectins [PEC]), sugar beet pulp [SBP], and wheat straw [WS]) on gas and total volatile fatty (VFA) production and molar proportions of individual VFA after 24 h *in vitro* incubation (n = 4 rabbits/treatment). Within each diet (low soluble fibre or high soluble fibre) and for each substrate, P_L and P_Q indicate lineal and quadratic effects ($P < 0.05$) of cellobiose, respectively. Bars indicate the standard error. (Experiment 6)

15 doses, respectively; Figure 11A), but had no effect with the LSF inoculum. For WS, cellobiose supplementation increased quadratically 24 h gas production with the LSF inoculum ($P = 0.001$; 24.3, 35.6 and 19.2 ml/g DM for 0, 7.5 and 15 doses, respectively; Figure 11A), but had no effect with the HSF inoculum. Donors' diet only affected the 24-h gas production for CEL substrate, being greater with the LSF than with the HSF inoculum (289 vs. 170 mmol/L; $P < 0.001$).

Interactions cellobiose $\times$ substrate ($P = 0.001$) and cellobiose $\times$ donors' diet ($P = 0.006$) were observed for total VFA production (Table 16). Whereas no effects ($P > 0.05$) of cellobiose or donors' diet were detected for GLU and SBP, cellobiose supplementation increased total VFA for CEL and PEC with both inocula ($P = 0.019$, quadratic for CEL; $P = 0.034$ and 0.016 , linear and quadratic for PEC), and decreased VFA production for WS with the HSF inoculum ($P = 0.003$, linear; Figure 11B). Total VFA production for CEL was greater with LSF than with HSF inoculum (20.7 vs. 12.9 mmol/L; $P = 0.014$), but the opposite was found for WS (3.97 vs. 6.21 mmol/L; $P = 0.005$).

The VFA profile differed widely among substrates, with CEL showing the lowest acetate ($P < 0.05$) and greatest butyrate ($P < 0.05$) proportions. Neither valerate nor caproate were detected in any sample, and no propionate, isobutyrate and isovalerate were detected in the fermentation of GLU. No effects ($P > 0.05$) of either cellobiose supplementation or donors' diet on VFA profile were observed for GLU, PEC, SBP, and WS substrates (Figure 11). In contrast, for CEL substrate cellobiose supplementation decreased isovalerate proportions with the HSF inoculum ($P = 0.007$, linear and 0.018 , quadratic), but had no effect with the LSF inoculum. Donors' diet only affected VFA profile for CEL substrate, with HSF-inoculated cultures showing greater acetate and isovalerate proportions (81.2 vs. 55.6 mol/100 mol, $P = 0.001$, and 1.04 vs. 0.07 mol/100 mol, $P = 0.026$, respectively) and lower

propionate proportions (13.6 vs. 42.6 mol/100 mol, $P < 0.001$) than those LSF-inoculated.

There were no relationships ($P > 0.05$) between either total VFA concentrations or VFA profile measured in the caecum of rabbits receiving the same experimental treatments (values reported in Experiment 1) and the values measured *in vitro* in the present study for CEL, GLU, SBP, and PEC substrates. However, a positive relationship ($r = 0.90$; $P = 0.015$; $n = 6$) was observed between total VFA caecal concentrations *in vivo* and *in vitro* when WS was used as substrate.

V. DISCUSSION

5.1. Influence of cellobiose supplementation and soluble fibre on fermentation, health, and growth traits in rabbits.

The addition of moderate levels of soluble fibre showed a positive effect on the performance and health of rabbits (Gómez-Conde et al., 2007 and 2009). This is in agreement with the results obtained in Exps. 1 and 2, as mortality was reduced in rabbits fed the HSF compared with those fed the LSF diet when rabbits received no CEL supplementation. It is accepted that LSF diets (with no or low level of medication) usually lead to high mortality rates in farms affected by ERE (Martínez-Vallespín et al., 2011; Trocino et al., 2013; Delgado, 2017). Nevertheless, the results could vary within the same farm along time as reported in this PhD thesis (Figure 12), suggesting that other factors than the diet influence the rabbit health status.

However, it was noticeable the interaction between CEL and soluble fibre detected for rabbit mortality (Figure 12). The supplementation of CEL (up to 7.5 g/L) in the drinking water of rabbits fed LSF diet reduced the mortality (Exps. 1, 2 and 3) (when it was high, > 20%, in the non-supplemented group) or at least did not increase it (when it was moderated in the non-supplemented group; Exp. 4), although it was not able to reduce mortality below the threshold of 10%. On the opposite, a higher level of CEL inclusion (15 g/L) combined with LSF diet increased the mortality (Exp. 1). In rabbits fed HSF diet, the supplementation of 7.5 and 15 g of CEL/L linearly increased the mortality (Exp. 1). This excess of CEL (15 g/L, equivalent to 2.2% in the feed; Exp. 1) negatively affected the rabbits' health at levels much lower than those reported by other authors in rats (15% in feed; Moinuddin and Lee, 1958). In contrast, the supplementation with 3.5 g of CEL/L to rabbits fed HSF diet tended to reduce the mortality in respect to 0.0 g of CEL/L when the mortality in the latter group was high (Exp. 2). These results might be related to the use of CEL by the intestinal microbiota as the increase in mortality in rabbits fed the HSF diet supplemented with CEL was parallel to a significant decrease of the caecal total VFA concentration, although this relationship was not observed for LSF7.5 group, but a change in the VFA profile (Exp. 1).

Figure 12. Influence of CEL supplementation on the mortality of growing rabbits. A) Mortality in animals fed with low soluble fibre diet (LSF); B) mortality in animals fed with high soluble fibre (HSF); **preW_0.0** referred to the group of rabbits offered no CEL before weaning and supplemented with it [preW_0.0- postW_7.0] or not [preW_0.0- postW_0.0] after weaning and fed with a low dietary soluble fibre level.

Cellobiose can be hydrolyzed in the small intestine by the enzyme lactase that has β -glucosidase and β -galactosidase activities (Dahlqvist, 1962; Nakamura, 2005; Morita et al., 2008). However, the intestinal lactase activity in weaned rabbits is extremely low (Marounek et al., 1995; Gutiérrez et al., 2002) and CEL hydrolysis may also be very slow (Fischer and Sutton, 1957). Therefore, it would be expected that most of the CEL was undigested and could be completely fermented by the intestinal microflora. Whether CEL was fermented before or in the caecum is not clear due to the lack of differences in the ileal (Exp. 1) and caecal pH (Exps. 1 and 4), although the differences found in VFA concentrations and profile in the ileum and caecum might suggest that CEL would be fermented in both segments. Accordingly, it would be expected that CEL produces some changes in the intestinal microbiota just after weaning, being a period where an important evolution of the intestinal microbiota occurs (Delgado, 2017). The two groups with lower mortality in Exp. 1 (HSF0-CEL and LSF7.5-CEL) showed also the highest butyrate proportions in the ileum, which may be related to a better integrity of the intestinal mucosa in the animals (Guilloteau et al., 2010) and/or changes in their microbiota metabolism and profile (Gantois et al., 2006; Song et al., 2013; Jiao et al., 2015). Intestinal VFA concentration measured at a single time point are the balance between the production rate and absorption, and cannot be considered as a good indicator of VFA production and/or absorption (Van der Klis and Jansman, 2002). Butyrate has shown higher absorption rates compared with acetate and propionate (Vernay, 1987; Von Engelhardt et al., 1989), and despite of this, butyrate proportion in the ileum was enhanced in the two groups with the lowest mortality (HSF0-CEL and LSF7.5-CEL) (Exp. 1). That might indicate the effect on the butyrate absorption being even stronger than the one observed on the ileal butyrate concentration.

Due to the difficulty to evaluate the butyrate production *in vivo*, an experiment to study the *in vitro* fermentation was performed. In this work, the butyrogenic effect of CEL was also confirmed in rabbits when *in vitro* fermentation of CEL was evaluated using inocula from rabbits fed LSF diet and supplemented with 7.5 g CEL/L or no CEL in the drinking water (Exp. 5 and 6). This butyrogenic effect of CEL was also reported in other species when it was fermented *in vitro* using a model system of the human colon (Van Zanten et al., 2012) or using pig feces as inoculum (Tran et al., 2016). Van Zanten et al. (2012) reported that CEL stimulated

the growth of *Lactobacillus acidophilus* NCFM and *Bifidobacterium animalis* subsp. *lactis* BI-04 under laboratory conditions. Furthermore, the supply of CEL with these two bacteria in an *in vitro* colonic model increased butyrate proportion and reduced the *Bacteroidetes/Firmicutes* ratio, whose high values have been linked with obesity and type 2 diabetes in humans.

In farm animals, the supplementation of cello-oligosaccharides (1.5 to 4.5 g/kg diet) to weaned pigs increased the *Lactobacillus* and decreased the *Clostridium* concentrations in jejunal contents (Jiao et al., 2014), and in broilers increased the viable counts of *Lactobacillus* in caecal contents and decreased the counts of *Escherichia coli* (Song et al., 2013). These results indicate that CEL supplementation can modify the intestinal microbiota which might be behind the shifts in the VFA profile observed in this PhD Thesis.

Nevertheless, the reduction of mortality might be also related to other factors. Rabbits from the groups with the lowest mortality in Exp. 1 (HSF0-CEL and LSF7.5-CEL) also showed a higher caproate proportion in the ileum. Caproate has shown antimicrobial activity by reducing *in vitro* the concentration of viable cells of an enteropathogenic *E. coli* strain (Skrivanova and Marounek, 2007), although the implication of *E. coli* in ERE is rather doubtful.

Furthermore, the negative influence of CEL supplementation on the health of rabbits fed HSF diet might be related to changes in the intestinal microbiota, as indicated by the reduction of butyrate and caproate proportions in the ileum and total VFA concentration in the caecum (Exp. 1). The latter might suggest a relevant negative effect of CEL on caecal fermentation of HSF fed rabbits, especially at the highest dose, and a potential dysbiosis. In fact, dietary CEL supplementation at higher doses (15% in feed) produced a general diarrhea in rats (Moinuddin and Lee, 1958), and sometimes at 10% dietary CEL (Umeki et al., 2004; Nishimura et al., 2010), suggesting an interaction of CEL with the host microbiota. The dietary supplementation with lactose up to 14% during the post-weaning period (which is hydrolyzed by the same enzymes that CEL) also showed a negative effect on the mortality of rabbits affected by ERE (Gutiérrez et al., 2002). All these results are in agreement with the highly variable response to the supplementation of different types of oligosaccharides as reviewed by Falcao-e-Cunha et al. (2007), which may be related to the dose used in the different studies and variations in the host microbiota.

Cellobiose has previously been reported to exert a positive effect by reducing inflammatory markers in mice with induced colitis, which was attributed to a prebiotic effect related to increased butyrate production (Nishimura et al. 2010). However, in Exp. 1, a pro-inflammatory response was observed in the appendix of rabbits fed the maximal dose of CEL supplementation (15 g/L), especially in those fed the HSF diet. These effects are in agreement with those obtained by supplementing a higher dose of CEL (15% in feed) to rats (Moinuddin and Lee, 1958), suggesting that the positive or negative interaction of CEL with the host microbiota and hence its prebiotic effects might be dose dependent. Moreover, at the time of sampling for the immune response analysis (46 d of age), the mortality in groups supplemented with 15 g of CEL/L was still increasing, which might account for the strong immune response observed, even when all the slaughtered rabbits were apparently healthy. The upregulation of the toll like receptors TLR-4 and TLR-2 in the appendix might indicate an activation of the innate immune response usually triggered by a pathogen associated molecular pattern (Williams, 2012). This would lead to the upregulation of the pro-inflammatory mediators such as TNF- α , iNOS and IL-6 (only in HSF group), which partially agrees with the cytokine profile previously reported in the appendix and caecal mucosa of rabbits affected by ERE (Bäuerl et al, 2014). The upregulation of MUC-1 in HSF fed rabbits might suggest a higher mucin secretion, although it also might play an anti-inflammatory role together with IL-10 in order to counterbalance the pro-inflammatory response (Sheng et al., 2013; Opal and DePalo, 2000). Therefore, the present results show the immune response in a precise moment (46 d of age; Exp. 1), and remark the importance of sampling time when characterizing the immune response in ERE affected rabbits in relation to the development of the disease and the interest to develop procedures that allow the evolution of the immune response along time.

Part of the mortality was produced once the experimental diet and CEL supplementation was withdrawn. This effect was more marked for HSF than for LSF group when CEL was no supplemented, which might be accounted for the change of the diet. The commercial diet supplied in this period was low in soluble fibre, as LSF diet (58.7 and 84.0 g/kg DM, respectively), but contained more insoluble fibre (341 and 307 g/kg DM, respectively). This might have altered the intestinal microbiota in HSF fed rabbits and increase the mortality at the end of the experimental period.

However, the increase of mortality once the CEL was withdrawn was even more evident in HSF groups indicating that the end of CEL supplementation might have also influenced in this upturn of the mortality. This agrees with the observations of the Exp. 4 where the mortality in rabbits fed a commercial diet and CEL in drinking water during all the experiment still increased relevantly once CEL was withdrawn.

The apparent negative effect of withdrawn CEL raised the question of how long the CEL treatment should last. It seemed that CEL supplementation for two weeks after weaning can be enough (Exps. 1 and 2); its extension for 10 more days did not provide better results in terms of mortality (Exp. 3). Moreover, CEL supplementation before weaning did not improve any parameters in the rabbit, but once the animals were weaned, the mortality rate was impaired (Exp. 4). However, there is no clear information available to help explain this effect, but it probably might be related to the microbial changes produced in the caecum in this period (Delgado, 2017).

The supplementation of moderate concentrations of CEL to rabbits fed LSF diet did not impair growth traits and punctually improved the weight gain and feed efficiency of the rabbits (Figure 13). It agrees with results in other species such as pigs and poultry where the supplementation of CEL improved growth performance (Otsuka et al., 2004; Song et al., 2013). However, in this work a reduction of feed intake or the improvement of feed efficiency during CEL supplementation would have been expected because of its additional energy supply, but these effects were not always observed (Figure 13A). The lack of a consistent effect might be related to a negative effect of CEL on the digestibility of other dietary components as fibre (not measured) and/or to the fact that CEL supplies the animal with VFA that provides a lower amount of energy than glucose, and part of that energy absorbed as butyrate might be used directly by the intestinal mucosa. In this context, the relative liver weight decreased with soluble fibre inclusion and no CEL supplementation (HSF0 vs. LSF0; Exp. 2) as previously reported (Falcao-e-Cunha et al., 2004; Pascual et al., 2014; Tazzoli et al., 2015), but also with the supplementation of CEL in rabbits fed a commercial LSF diet (Exp. 4). Papadomichelakis et al. (2012) observed a reduction of liver weight combined with reduced hepatic glycogen and cholesterol concentrations. They related it to lower postprandial glucose levels due to the lower starch level when the fermentable fibre increased. In broilers, similar effects on the

weight of liver and postprandial glucose levels were reported when fed pea substituted wheat (Classen et al., 2017).

The evaluation of the *in vitro* fermentation also provided additional information about the caecal fermentation in rabbits. In agreement with previous results in pigs and ruminants showing the influence of the diet of donors on *in vitro* fermentation of different substrates (Bindelle et al., 2007; Mateos et al., 2013), the production of both gas and VFA in the blanks (Exp. 6) was strongly influenced by the CEL supplementation. In contrast, there was no effect of the soluble fibre and no CEL $\times$ donors' diet interaction was detected (Exp. 6). The lack of influence of the soluble fibre content on fermentation in blanks was unexpected, especially taking into account the positive influence exerted by the inclusion of soluble fibre in the diet on fibre digestibility, both at ileal and faecal level, observed previously by our group (Abad-Guamán et al., 2015) and in the microbial profile (Gómez-Conde et al., 2007, 2009).

However, donors' diet affected both gas production parameters and VFA profile when substrates were incubated (Exp. 6). The significant CEL $\times$ donors' diet interactions detected for several parameters suggested differences in the fermentative activity of LSF and HSF inocula, which might be related to different microbial communities in both inocula. In fact, Gómez-Conde et al. (2009) observed differences in caecal microbiota diversity in rabbits fed diets with soluble fibre contents (79 to 131 g soluble fibre/kg DM), similar to those used in our study. Furthermore, the increase of dietary soluble fibre led to a higher *in vivo* ileal and caecal total VFA (Exp. 1), although this effect was not observed *in vitro* (Exp. 6). These *in vivo* results agree with the increase of the ileal and faecal digestibility of fibre (both soluble and insoluble) when the soluble fibre level increased (Abad-Guamán et al., 2015), but it was not observed in the caecum. As hypothesized by Abad-Guamán et al. (2015), it might suggest that the cell wall polysaccharides in the ileum can be hydrolyzed to carbohydrates with lower molecular weight (to shorter polysaccharides or to oligosaccharides) and partially, but not extensively, fermented. In fact, the molecular weight of β -glucan of oats was reduced after passage through the stomach and the proximal small intestine of pigs. It was probably associated to the microbial enzymes, although this activity did not lead to a loss in β -glucan from the digesta before the lower small intestine was reached (Johansen et al., 1993).

Figure 13. Influence of supplement CEL on the growth performance of growing rabbits fed with low soluble fibre (LSF) or high soluble fibre (HSF) diets. A and B) Average daily feed intake (ADFI); C and D) Average daily weight gain (ADG); E and F) Feed efficiency (G:F); **preW_0.0** referred to the group of rabbits offered no CEL before weaning and supplemented with it [preW_0.0- postW_7.0] or not [preW_0.0- postW_0.0] after weaning.

Consequently, low molecular weight carbohydrates derived from microbial fibre hydrolysis would be considered as fermented in the ileum (because they would not be precipitated with ethanol), although they could be mostly fermented in the caecum.

This result may be explained by the fact that some bacteria possesses polysaccharide depolymerases but not glycoside hydrolases necessary for utilization of the xylo-oligosaccharides or oligogalacturonides as an energy source leading to the solubilization but not fermentation of cell wall polysaccharides (Dehority, 1993). In this way, Fondevila and Dehority (1994) demonstrated that ruminal hemicellulose utilization resulted from initial solubilization of the hemicellulose from the forage by non-hemicellulose utilizers and subsequent fermentation of this soluble polysaccharide by the utilizing, but non-degrading, bacteria. This circumstance may occur in rabbits fed HSF diet and may affect the ileal and caecal microbiota profile and gut health.

The CEL as substrate, fermented *in vitro* was the most affected substrate by both CEL supplementation and donors' diet (Exp. 6). Cellobiose supplementation decreased *Lag* and *Ti* values, indicating that fermentation of CEL started earlier with the inocula from rabbits receiving CEL in the drinking water. This could be due to an adaptation of the caecal microbiota to CEL fermentation during the seven days-period of diet adaptation before conducting the *in vitro* trial. In addition, the fermentation of CEL with the LSF inoculum generated more total VFA than with the HSF inoculum (20.7 vs. 12.9 mmol/L), and increased butyrate (42.6 vs. 13.6 mol/100 mol) at expenses of acetate (55.6 vs. 81.2 mol/100 mol). These results agree with the observation of Yang et al. (2010) where the fermentation of CEL as substrate with rabbits' inoculum resulted in an increment of the total VFA and butyrate proportion produced. Furthermore, *in vitro* fermentation of CEL using pig faeces as inoculum tended to increase the proportion of butyrate compared with xylo-oligosaccharides, but also the microbiota profile after 12 and 24 h of fermentation, where the counts of *Bacteroides* increased with CEL with respect to xylo-oligosaccharides (Tran et al., 2016). The results obtained in Exp. 6 would help to explain the positive effects of the LSF7.5 treatment on rabbit's health previously discussed (Exp. 1).

However, our *in vitro* results (Exp. 6) cannot explain the negative effects of CEL supplementation on mortality of rabbits fed the HSF diet observed in Exp. 1. It might be only hypothesized a potential negative effect of CEL on the intestinal microbiota of HSF fed rabbits, or an unknown mechanism explaining the low mortality found with both HSF0 and LSF7.5 treatments. However, the *in vitro* fermentation profile of CEL with HSF0 and LSF7.5 inocula differed widely, both quantitatively (7.23 vs. 26.1 mmol total VFA/L) and qualitatively (48.1 vs. 87.0, and 47.8 vs. 8.47 mol/100 ml for acetate and butyrate proportions, respectively) which might indicate that the reduction of mortality in these two treatments is derived from different causes. It should be noted that our *in vitro* study (Exp. 6) was conducted using soft faeces as inoculum and the possible effects of CEL supplementation on other parts of the digestive tract could not be assessed. Therefore, it would be interesting to analyze whether the use of inoculum from the small intestine, where at least part of the CEL can be fermented, produces similar results to those observed in this study using soft faeces or not.

5.2. Modification of the *in vitro* fermentation methodology.

Finally, as part of this PhD Thesis, Exp. 5 was performed in order to improve the methodology of the *in vitro* caecal fermentation in rabbits. As commented previously, some studies have been used the *in vitro* gas production technique (Menke et al., 1979) to investigate caecal fermentation in rabbits, where differences in the substrate processing have been observed among studies. The problem that exist about the loss of the soluble fibre during the filtration/centrifugation process when an enzymatic pre-digestion is done in studies using pig or rabbit material as inoculum (Bauer et al., 2003; Bindelle et al., 2007ab; Rodríguez-Romero et al., 2011) can be solved by avoiding the filtration/centrifugation step and using the whole material (solid and liquid) obtained after the pre-digestion procedure as substrate for *in vitro* caecal incubations. However, using this alternative, the enzymes added in the pre-digestion procedure could be fermented and contribute to gas and VFA production (Carro et al., 2005). The influence of a pre-digestion procedure on the *in vitro* caecal fermentation in rabbits has not yet being investigated to our knowledge. Additionally, the lack of a filtration/centrifugation step would permit that glucose and amino acids released from starch and protein hydrolysis be finally fermented,

which would be an unwanted effect. However, the use of this procedure with substrates with low or even no content of starch and protein could limit this effect.

In this Experiment, the greater gas production in the pre-digested blanks than in those untreated was attributed to the fermentation of the added enzymes. Carro et al. (2005) analyzed the effects of treating several substrates with fibrolytic enzymes on *in vitro* ruminal fermentation, and also observed greater gas production in the blanks including the fibrolytic enzymes. These authors also noticed that using different blanks (with or without enzymes added) for correcting gas production values altered the differences between experimental treatments and changed the interpretation of results. The increased total VFA production and the shifts in VFA profile observed in our study when the blanks were pre-digested also indicated a fermentation of enzymes, as the amino acids resulting from proteolysis can be deaminated and the generated carbon skeletons be fermented to VFA (Van Soest, 1994; Vanegas et al., 2017). The greater isovalerate proportions detected in the pre-digested blanks are consistent with this hypothesis, because this VFA generally arise from the fermentation of leucine (Wallace and Cotta, 1988). Therefore, both gas and VFA production values in our study were corrected for the amount of gas and VFA, respectively, produced in the corresponding blanks. The inclusion of enzymes also increased the proportion of butyrate, that quantitatively is the second VFA in the rabbit caecum (García et al., 2002a).

The effect of both the pre-digestion and donors' diet on gas and VFA production depended on the incubated substrate (Figures 7 and 8; Exp. 5). The pre-digestion had no influence on the *in vitro* gas production kinetics of WS, and had only a moderate effect on that of SBP (reduction of lag time), although in both cases the pre-digestion decreased the molar proportion of acetate and, in combination with the donors' diet, modified total VFA production for WS. The similarity in the results observed for SBP and WS contrasts with the different proportion of solubilized carbohydrate fraction obtained after the pre-digestion procedure (40 and 22%, respectively) previously reported by Abad et al. (2013) for the same substrates. The lack of a stronger influence of the pre-digestion on the fermentation of these substrates might be explained by a quick solubilization of this carbohydrate fraction when the untreated WS and SBP were mixed with the inoculum. Using a different experimental approach (pig faeces as inoculum) and the insoluble residue of the

pepsin-pancreatin pre-digestion of SBP (and other carbohydrate-rich substrates) as substrate, Bauer et al. (2003) reported lower *in vitro* gas production for pre-digested than for untreated substrates. However, it should be noticed that these authors obtained the residue of the pre-digestion by centrifugation, and therefore the soluble potentially fermented compounds were lost, which might partly account for the different results obtained in two studies. In our study, the substrate was not centrifuged/filtered after the pre-digestion, and therefore contained all generated compounds (mainly soluble fibre, but also protein, especially in SBP) that could be further fermented by the caecal microbiota. However, no increase in either gas or VFA production was observed by the pre-digestion of SBP and WS. Contrary to the results of Bauer et al. (2003), the use of the pre-digested insoluble residue (excluding by filtration the soluble fraction) of SBP did not modify the *in vitro* gas production compared with the untreated one, using rabbit caecal digesta (Kermauner and Lavrenčič, 2013). These results might indicate a minor influence of the solubilized fraction in the pre-digestion on the *in vitro* fermentation kinetics. Differences either in the *in vitro* solubility of SBP or in the caecal inoculum can also help to explain the controversial results observed in different studies.

The CEL was the most affected substrate by both the pre-digestion and the donors' diet (Figures 7 and 8; Exp. 5). The results indicate that the combination of pre-digestion and LSF-inoculum caused an increase in the amount of CEL fermented, as cumulative gas production is directly related to the organic matter fermented (Menke et al., 1979); however, this effect was not observed for the other substrates. The increased total VFA production observed when CEL was pre-digested (mainly with the LSF inoculum) contributes to strength this hypothesis. These results might be explained by an eventual modification of CEL structure due to the pre-digestion procedure and/or a potential synergy between the added enzymes and CEL, combined with a specific interaction with the microbiota present in the LSF-inoculum. There are some evidences about the potential modification of CEL by the pre-digestion procedure, as CEL can be hydrolyzed at low pH values (2.0 – 3.0) such as that used in the first step of the pre-digestion procedure (Bobleter et al., 1986). Moreover, CEL can be extensively digested in the small intestine of rats by a β -galactosidase present in the mucosal membrane (Morita et al., 2008), and the presence of cellobiosidase in the intestine of herbivores has been previously

described (Nakamura, 2005). However, it is not probable that the pancreatin used in our study had these enzymatic activities, although this point would require further confirmation. The combination of added enzymes and CEL might have interacted specifically with the microbiota of the LSF-inoculum, as suggested by the marked shifts observed in the proportions of acetate and butyrate. In agreement with our results, Falcão-e-Cunha et al. (2004) found that caecal fibrolytic activity in rabbits depended on the type of dietary fibre. Others have also reported that a reduction of the dietary level of soluble fibre reduced the ileal and faecal digestibility of different fibrous fractions (Abad-Guamán et al., 2015) and modified the caecal microbiota of rabbits (Gómez-Conde et al., 2007 and 2009). The microbial communities in the LSF-inoculum might have also interacted with the pre-digested CEL, causing changes in VFA profile. A change of butyrate for acetate also resulted from the caecal *in vitro* fermentation of untreated CEL using rabbit caecal contents obtained from donors' rabbits fed with a low soluble fibre diet (Yang et al., 2010). All these results might indicate that the amount of fermentable substrate available for the microbiota would have been lower for the LSF compared with the HSF diet, which in turn might have influenced the activity and/or adaptability of the microbiota. An adaptation of the microbiota to CEL seems to be required according to the large lag time values observed for this substrate (25.3 h), although the time for maximal rate of gas production (T_i) was similar to that for PEC and lower than that for SBP. It seems that a synergy occurred when the pre-digested CEL was incubated with the LSF-inoculum, which might be related to the potentially high demand of amino acids at initial stages of fermentation, thus avoiding the use of amino acids as energy source. The lack of isovalerate production detected for the pre-digested CEL with the LSF-inoculum (although not different from the other treatments) would support this hypothesis.

Contrary to that observed for CEL, k and μ_m values increased when pre-digested PEC was fermented by the HSF-inoculum compared with the untreated PEC, but there was no effect with LSF-inoculum. This might be related to a better adaptation of the microbiota from the HSF-inoculum to the pre-digested PEC and/or a potential modification of PEC by the pre-digestion, as suggested by the PEC flocculation observed under the acidic conditions of the first step of the pre-digestion. These results are in agreement with the changes previously observed by

Tran et al. (2016) in the gas production, VFA molar proportions and microbiota profile when mucins (another fraction of the endogenous substances) were added to *in vitro* batch fermentations, although only minor interactions were detected between mucins and the incubated substrates (cellulose and inulin). Further studies would be required to study whether the supplementation of the substrate with other quantitatively relevant endogenous substances (like mucins) should be implemented, as previous *in vivo* and *in vitro* studies have reported a high fermentability of mucins (Marounek et al., 2000; Abad-Guamán et al., 2015).

Based on the results discussed for Exp. 5, the pre-digestion of the substrate before the *in vitro* caecal fermentation is recommended in order to exert potential changes in the substrate similar to those produced during the digestion in the stomach and small intestine and to provide some endogenous substances for caecal fermentation. The level of soluble fibre in the donors' diet also influences the *in vitro* caecal fermentation but its effect was strongly dependent on the type of substrate.

In addition to the rather similar results obtained *in vivo* and *in vitro* for molar proportion of butyrate, minor similarities were found between *in vivo* and *in vitro* fermentation, in spite of the experimental factors influenced the *in vitro* fermentation of some substrates. Only a significant relationship between caecal VFA concentrations *in vivo* and *in vitro* was observed when WS was used as substrate. The different conditions in rabbit's caecum and *in vitro* (i.e. buffer capacity of the incubation medium, ratio substrate/incubation medium, digesta retention time, movements, substrate composition, etc.) can explain the lack of significant *in vivo-in vitro* relationships. It should be also considered that VFA are removed from the caecum by absorption and flow to the colon, whereas there is no absorption or digesta flow in the *in vitro* cultures. In addition, pure substrates were incubated *in vitro*, whereas non-digested fractions of feeds are the materials potentially fermented in the caecum. The conditions in the *in vitro* cultures may have caused a selection of some bacterial strains, as it has been previously shown in *in vitro* cultures inoculated with fresh faeces from pigs (Boudry et al., 2012) and with ruminal fluid (Mateos et al., 2015). The latter might account for the differences in the VFA profile obtained, like the lack of caproate found in the *in vitro* fermentation. Furthermore, the presence of a wide type of endogenous substances in the caecal *in vivo* fermentation might contribute to these differences. In fact, when the caecal *in vitro* fermentation was

done with no pre-digestion (and accordingly no enzymes) the average molar proportion of acetate was 99%, a value closer to the acetate proportion found in the ileum (94%) than in the caecum (84%). However, when a pre-digestion of the substrate was done the acetate proportion decreased up to 87%, a value closer to that found *in vivo* in the caecum. This indicates the importance to supplement the culture medium for *in vitro* caecal fermentation with endogenous substances, as suggested by other authors. All these results would support that the *in vitro* fermentation would emulate qualitatively and no quantitatively the *in vivo* fermentation (Williams et al., 2005).

VI. COCLUSIONS AND IMPLICATIONS

6.1. CONCLUSIONS

1. The rabbits' health status depended on both the dietary soluble fibre level and on the cellobiose supplementation.
  - Doses of 7.0 and 7.5 g of cellobiose/L in the drinking water decreased the mortality when the rabbits where fed with diets low in soluble fibre content.
  - Positive effects on mortality might be related with the increase of butyrate proportion observed in the ileal fermentative profile *in vivo* and in the caecal fermentation *in vitro*.
2. The effect of cellobiose supplementation and dietary soluble fibre on the *in vitro* caecal fermentation was highly dependent on the substrate.
3. A pre-digestion of the substrate is recommended for *in vitro* fermentation.

6.2. IMPLICATIONS

As a consequence of the results obtained in the present PhD Thesis, the inclusion of moderate concentrations of cellobiose (≤ 7.5 g/L) in the drinking water of rabbits fed a low soluble fibre diet would be recommended to control mortality rate when no antibiotic is supplied and considering that cellobiose would be economically profitable. It would be interesting to evaluate the synergic potential of the cellobiose with other beneficial oligosaccharides (e.g., xylo-oligosaccharides) in order to reduce mortality below the threshold of 10%. Cellobiose concentrations higher than 7.5 g/L are not recommended, independently of the dietary soluble fibre level.

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ANNEX

Annex 1. Effect of pre-digestion, type of diet fed to donors' rabbits of soft faces (low soluble fibre or high soluble fibre) and incubated substrate on gas production rate in 144 h of *in vitro* incubations (n = 4 rabbits/treatment). (*Experiment 5*)

Low soluble fibre
0.0 g of cellobiose/L

High soluble fibre

7.5 g of cellobiose/L

15.0 g of cellobiose/L

● Cellobiose   ○ Glucose   ● Pectins   ■ Sugar beet pulp   □ Wheat straw

Annex 2. Effect of the concentration of cellobiose (0.0, 7.5 and 15 g/L), type of diet fed to donors' rabbits of soft faces (low soluble fibre or high soluble fibre) and incubated substrate on gas production rate in 144 h of *in vitro* incubations (n = 4 rabbits/treatment). (Experiment 6)

Annex 3. Effect of the concentration of cellobiose (0.0, 7.5 and 15 g/L), type of diet fed to donors' rabbits of soft faces (low soluble fibre or high soluble fibre) and incubated substrate on gas kinetics in 48 h of *in vitro* incubations (n = 4 rabbits/treatment. Standard error = 8.86 ml/g DM substrate). (*Experiment 6*)