INTERNATIONAL MASTER ON ANIMAL BREEDING AND REPRODUCTION BIOTECHNOLOGY

Influence of the metagenome on resistance to stress and diseases in rabbits

Master thesis

Valencia, September 2020

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Acknowledgments

I am very grateful to my supervisors Agustin Blasco and Maria josé Argente for their guidance, time, efforts, and teaching. They created a critical sense in me and taught me how to work rigorously. It was an honour working with you and I couldn't have asked better supervisors.

I would like to express my gratitude to the Mediterranean Agronomic Institute of Zaragoza (IAMZ) and the International Centre for Advanced Agronomic Mediterranean Studies (CIHEAM) for funding my studies during these two years of the Master.

I also would like to thank all my colleagues in the department, especially to Houda, Ayman, Monica, Vicky, Cristina, Agustina and Samuel for all the time we spent together.

I also want to thank my parents, my family and my HK for being always by my side despite the distances. I feel very lucky to have you.

Abstract

In recent years, the environmental variance has aroused great interest in animal genetics. Environmental variance has been related to the animal's capacity to adapt to new environmental challenges, i.e., animal welfare. Several studies have reported that environmental variance is under genetic control. This has implications for livestock production, since selection to reduce environmental variance may be a useful way to decrease the susceptibility to diseases and stress in animals, i.e., to improve animal welfare. Concerning susceptibility to diseases and stress, it is well known that gut microbiota has an important role in the development of the immune system and the activation of the hypothalamic-pituitary-adrenal (HPA) axis in the host. A divergent selection experiment for the environmental variance of litter size at birth was carried out in rabbits during thirteen generations. The aims of this study were to analyse theinflammatory response of the high and the low line in order to study the effect of selection for the environmental variability of litter size on susceptibility to diseases, and to analyse the existence of microbial genes associated with the environmental variability of litter size that allows us to separate the high and the low line. For these purposes, several inflammatory and biochemical markers were measured in 39 females from the high and the low line, and partial least square discriminant analysis (PLS-DA) was performed using 34 females from the high line and 36 females from the low line. All animals belonged to the 13^{th} generation of selection.

The high line showed lower white blood leukocyte count (WBC) (-0.87 $x10^3$/µl) and percentage of basophils (-0.11%) and higher concentration of TNF-α (+13.8 pg/ml),C-reactive protein (CRP) (+38.1µg/ml), bilirubin (+0.08 µmol/L), cholesterol (+0.14 µmol/L), gamma-glutamyl transferase (GGT) (+0.35 U/L) and alkaline phosphatase (ALP) (+2.4 U/L) compared to the low line. Therefore, the high line showed higher inflammatory response and susceptibility to infectious and diseases.

Metagenomic analysis was carried out on the animals of previous study. Bacterial genome sequencing was performed by paired-end (2x150) in Illumina NextSeq 550. A total of 3046 microbial genes were identified. The PLS-DA score plot showed that the projection of data on the four latent variables had a good adjustment coefficient (R^2=0.85) and a good cross-validation (Q^2=0.52). A good separation was observed between lines and, the loading plot shows how the variables relate to each line and that the two lines are negatively correlated.

In conclusion, our findings suggest that selection for the environmental variability of litter sizeis related to susceptibility to diseases in animal through modification of its gut microbiota.

Keywords: C-reactive protein, divergent selection response, rabbits, susceptibility to diseases, welfare, metagenomic, PLS-DA.

Resumen

En los últimos años, la varianza ambiental ha despertado un gran interés en la mejora genética animal. La varianza ambiental se ha relacionado con la capacidad del animal para adaptarse a nuevos desafíos ambientales, es decir, con el bienestar animal. Varios estudios han encontrado que la varianza ambiental está bajo control genético. Esto tiene implicaciones para la producción ganadera, ya que la selección para reducir la varianza ambiental puede ser una forma útil de disminuir la susceptibilidad a enfermedades y estrés en los animales, es decir, sería una forma útil de mejorar el bienestar animal. En cuanto a la susceptibilidad a las enfermedades y al estrés, la microbiota intestinal tiene un papel importante en el desarrollo del sistema inmunológico y la activación del eje hipotalámico-pituitario-adrenal (HPA) en el huésped. Se llevó a cabo un experimento de selección divergente para la varianza ambiental del tamaño de la camada al nacimiento en conejos durante trece generaciones. Los objetivos de este estudio fueron analizar la respuesta inflamatoria de la línea alta y baja con el fin de estudiar el efecto de la selección por variabilidad ambiental del tamaño de la camada sobre la susceptibilidad a enfermedades, y detectar genes microbianos asociados con la variabilidad ambiental del tamaño de camada que nos permitan separar la línea alta y la baja. Para ello, se midieron varios marcadores inflamatorios y bioquímicos en 39 hembras de la línea alta y baja, y se realizó un análisis discriminante de mínimos cuadrados parciales (PLS-DA) utilizando 34 hembras de la línea alta y 36 hembras de la línea baja. Todos los animales pertenecían a la 13ª generación de selección.

La línea alta mostró menor recuento de leucocitos en sangre (WBC) (-0,87 x103 / µl) y porcentaje de basófilos (-0,11%) y mayor concentración de TNF-α (+13,8 pg / ml), proteína C reactiva (PCR) (+ 38,1 µg / ml), bilirrubina (+0,08 µmol / L), colesterol (+0,14 µmol / L+0,14 µmol / L), gamma-glutamil transferasa (GGT) (+0,35 U / L) y fosfatasa alcalina (ALP) (+2,4 U / L) en comparación con la línea baja. Por lo que la línea alta mostró una mayor respuesta inflamatoria y susceptibilidad a enfermedades infecciosas.

El análisis metagenómico se llevó a cabo en los animales del estudio anterior. La secuenciación del genoma bacteriano se realizó por paired-end (2x150) en Illumina NextSeq 550. Se identificaron un total de 3046 genes microbianos. Los resultados del PLS-DA mostraron que la proyección de los datos sobre las cuatro variables latentes tuvieron un buen coeficiente de ajuste ($R^2 = 0.85$) y de validación cruzada ($Q^2 = 0.52$). Se observó una buena

separación entre líneas y, la gráfica mostro cómo las variables se relacionan con cada línea y que las dos líneas están correlacionadas negativamente.

En conclusión, nuestros resultados sugieren que la selección por varianza ambiental del tamaño de camada está relacionada con la susceptibilidad a enfermedades en el animal a través de la modificación de su microbiota intestinal.

Palabras clave: proteína C reactiva, respuesta de selección divergente, conejos, susceptibilidad a enfermedades, bienestar, metagenómica, PLS-DA.

Resum

En els últims anys, la varianza ambiental ha despertat un gran interés en la millora genètica animal. La varianza ambiental s'ha relacionat amb la capacitat de animal per a adaptar-se a nous desafiaments ambientals, és a dir, amb el benestar animal. Diversos estudis han trobat que la varianza ambiental està baix control genètic. Açò té implicacions per a la producció ramadera, ja que la selecció per a reduir la varianza ambiental pot ser una forma útil de disminuir la susceptibilitat a malalties i estrés en els animals, és a dir, seria una forma útil de millorar el benestar animal. Quant a la susceptibilitat a les malalties i a l'estrés, la microbiota intestinal té un paper important en el desenrotllament del sistema immunològic i l'activació de l'eix hipotalámico-pituitario-adrenal (HPA) en l'hoste.

Es va dur a terme un experiment de selecció divergent per a la varianza ambiental de la grandària de la ventrada al naixement en conills durant tretze generacions. Els objectius d'este estudi van ser analitzar la resposta inflamatòria de la línia altai baixa a fi d'estudiar l'efecte de la selecció per variabilitat ambiental de la grandària de la ventrada sobre la susceptibilitat a malalties, i detectar gens microbians associats amb la variabilitat ambiental de la grandària de ventrada que ens permeten separar la línia alta i la baixa. Per a això, es van mesurar diversos marcadors inflamatoris i bioquímics en 39 femelles de la línia alta i baixa, i es va realitzar una anàlisi discriminant de mínims quadrats parcials (PLS-DA) utilitzant 34 femelles de la línia alta i 36 femelles de la línia baixa. Tots els animals pertanyien a la 13a generació de selecció.

La línia alta va mostrar menor recompte de leucòcits en sang (WBC) (-0,87 x103 /µl) i percentatge de basòfils (-0,11%) i major concentració de TNF (+13,8 pg /ml) , proteïna C reactiva (PCR) (+ 38,1 µg / ml) , bilirubina (+0,08 µmol / L), colesterol (+0,14 µmol / L+0,14 µmol / L) , gamma-glutamil transferasa (GGT) (+0,35 U / L) i fosfatasa alcalina (ALP) (+2,4 U / L) en comparació amb la línea baixa. Pel que la línia alta va mostrar una major resposta inflamatòria i susceptibilitat a malalties infeccioses.

L'anàlisi metagenómico es duc a terme en els animals de l'estudi anterior. La seqüenciació del genoma bacterià es va realitzar per paired-end (2x150) en Illumina NextSeq 550. Es van identificar un total de 3046 gens microbians. Els resultats del PLS-DA van mostrar que la projecció de les dades sobre les quatre variables latents van tindre un bon coeficient d'ajust (R^2 = 0.85) i de validació encreuada (Q^2 = 0.52). Es va observar una bona separació entre

línies i, la gràfica va mostrar com les variables es relacionen amb cada línia i que les dos línies están correlacionades negativament.

En conclusió, els nostres resultats suggerixen que la selecció per varianza ambiental de la grandària de ventrada està relacionada amb la susceptibilitat a malalties en l'animal a través de la modificació del seu microbiota intestinal.

Paraules clau: proteïna C reactiva, resposta de selecció divergent, conills, susceptibilitat a malalties, benestar, metagenómica, PLS-DA.

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Chapter 1. Introduction

1. Part 1

1.1. Environmental variation

1.1.1. Environmental variation in quantitative traits

The value observed or the phenotypic value of an individual can be partitioned into two components, one attributable to the genotype and one attributable to the environment.

$$P = G + E \quad (1)$$

where P is the phenotypic value, G is the genotype value and E is the environmental value. We assume that the genotype conferring a certain value on the individual, and the environment causing a deviation from this value.

Additionally, the formula (1) can include the genotype and environmental interaction (G x E); for example, when the best genotypes in certain environments are not necessarily the best in other environments. In intensive production systems as the poultry, the pigs, the rabbits and the dairy cattle, the facilities and the animal care are very similar regardless of the place of production. Therefore, the genotype-environment interaction is not considered a major problem and it is often ignored in these species.

When the genotype-environment interaction is not considered,the phenotypic variation (V_P) within a population can be divided into genetic variation (V_G) and environmental variation (V_E). This relationship can be summarized as follows (Falconer and Mackay, 1996):

$$V_P = V_G + V_E \quad (2)$$

The genetic variation (V_G) includes the variance due to the average additive value of the genes and the variance due to values of the intra and inter-locus interactions among genes. The environmental variation (V_E) can be divided into two components, the variance due to systematic recognisable environmental causes, as season, feed, age or sex effects, and the variance due to unknown or random environmental causes that therefore cannot be eliminated. Note that there is an important assumption behind the formula (2) as the genotype and the environment are not correlated.

A reduction in the environmental variance would lead to increase the heritability (h^2) of trait, which can be particularly helpful for increasing the response to selection in traits with low heritability.

Environmental variance can be reduced by control of systematic environmental effects, i.e., making them as uniform as possible, by pre-correction of the data, or by selection. The control of systematic environmental effects is difficult to carry out in species under extensive production systems as beef cattle, sheep, and goats. The pre-correction of the data can be a rude way for eliminating environmental variance, if effect acts over a long period as in the case of season effect on litter size, since the response in each animal can be wide variable. Finally, the response to selection is conditioned to heritability. In order to analyse the genetic determination of environmental variability, several studies have been performed in the last decade.

1.1.2. Genetic control of environmental variation

The response found in a direct selection experiment for litter size environmental variance in rabbits by Blasco et al. (2017) confirmed the genetic control in environmental variance, in agreement to heritability estimated in previous studies with pigs (Sorensen and Waagepetersen, 2003 for litter size; Ibañez-Escriche et al., 2008a for slaughter weight; Mulder et al., 2015 for birth weight and stillbirth; Sell-Kubiak et al., 2015 for birth weight), rabbits (Garreau et al., 2008 for birth weight; Yang et al., 2011 for litter size), mice (Gutiérrez et al., 2006 for litter size; Ibañez-Escriche et al., 2008b for weight gain; Formoso-Rafferty et al., 2015 for birth weight), poultry (Mulder et al., 2009 for body weight), beef cattle (Fina et al., 2013 for birth weight) and dairy cattle (Rönnegård et al., 2013 for milk yield). Note that all of these studies provide indirect evidence about the genetic determination of environmental variance, since these estimates come from analyses of databases and not from experiments designed to assess the genetic determination of environmental variance.

All these studies show that there is additive variance but the heritability of environmental variance is low (see Table 1). All of them find the existence of a genetic correlation ($r_A(a,a_v)$) between additive genetic effects for the trait (a) and its environmental variance (a_v), although the majority of estimates present awide confidence interval. The correlated response to selection for environmental variance on the mean of a trait depends on the value and the sign of this correlation. A negative genetic correlation means that when we select to increase the

environmental variability, the mean of this trait decreases and, vice versa, a positive genetic correlation means that when we select to increase the environmental variability, the mean of this trait increases.

Table 1 shows a negative genetic correlation between litter size and its environmental variance (Sorensen and Waagepetersen, 2003; Gutiérrez et al., 2006). In agreement with the negative genetic correlation between both traits, Blasco et al. (2017) found a higher litter size in the selected line for decreasing litter size variability than for increasing it in rabbits. Body weight does not show a clear pattern (Table1), some authors have found a negative correlation between the trait and its environmental variability (Ibañez-Escriche et al., 2008a; Ibañez-Escriche et al., 2008b; Mulder et al., 2009), and others have reported a positive genetic correlation (Fina et al., 2013; Sell-Kubiak et al., 2015). Indeed, Formoso-Rafferty et al. (2015) have found a higher body weight at birth in the selected line for increasing the environmental variance of body weight at birth in mice than in the selected line for decreasing it.

Table 1. Estimates of the heritability of environmental variance (h_v^2), additive variance ($\sigma^2_{a_v}$) and genetic correlation between additive genetic effects for the trait and its environmental variance ($r_A(a,a_v)$).

Source	Trait	h_v^2	$\sigma^2_{a_v}$	$r_A(a,a_v)$
Sorensen and Waagepetersen (2003)	Litter size pigs	0.026	0.09	-0.62
Gutiérrez et al. (2006)	Litter size mice	0.048	0.18	-0.93
Ibañez-Escriche et al. (2008a)	Weight gain mice	0.018	0.20	-0.19
Ibañez-Escriche et al. (2008b)	Slaughter weight pigs	0.011	0.11	-0.07
Garreau et al. (2008)	Birth weight rabbits	0.013	0.06	_
Mulder et al. (2009)	Body weight broiler males	0.046	0.24	-0.45
	Body weight broiler females	0.047	0.32	-0.41
Yang et al. (2011)	Litter size rabbits	0.041	0.14	-0.73
Fina et al. (2013)	Birth weight beef cattle	0.130	0.55	0.44
Rönnegård et al. (2013)	Milk yield dairy cattle	0.003	0.05	0.60
Sell-Kubiak et al. (2015)	Birth weight pigs Landrace	0.011	0.06	0.55
	Birth weight Large White	0.008	0.05	0.62
Blasco et al. (2017)	Litter size rabbits	0.08	0.21	-0.06

1.2. Selection experiments for environmental variation in animals

In recent years, the environmental variance has aroused great interest in animal genetic improvement (Hill and Mulder, 2010). Reducing the environmental variance in traits with low heritability has the advantage of improving the response to selection since it increases the heritability. Moreover, as we will see later, the reduction of environmental variance is related to the improvement of animal welfare. Besides, reducing the environmental variance can increase the homogeneity of productions, which is a specificity preferred by farmerssince it optimizes handling and increases economic efficiency.

Four divergent selection experiments for environmental variance have been carried out, two in rabbits (Blasco et al., 2017; Garreau et al., 2008), one in mice (Formoso-Rafferty et al., 2015), and one in pigs (Larzul et al.,2006).

In rabbits, Blasco et al. (2017) reported a successful divergent selection for the environmental variance of litter size over ten generations. The environmental variance of litter size was calculated as the within-doe variance of litter size after pre-correctinglitter size for both year-season and parity-lactation status. The high line showed a higher variability (4.4 kits^2) than the low line (2.7 kits^2). This selection also showed differences between the divergent lines for reproductive traits such as litter size; the low line has a higher litter size (8.3 kits) than the high line (7.7 kits) (Agea et al., 2019), due to more advanced embryonic development (Calle et al., 2017) and higher implanted rate (Argente et al., 2017). As another correlated response, the high line has a higher sensitivity to diseases and less tolerance to stress (Argente et al., 2019). Another divergent selection experiment in rabbits was based on the homogeneity of birth weight (Garreau et al., 2008). After 10 generations of selection, the standard deviation of birth weight was (7.34 g) in the homogeneous line against 11.26 g in the heterogeneous line (Bodin et al., 2010a). Besides, the mortality of young rabbits between birth and weaning was lower in the homogeneous line than in the heterogeneous one (17.7% vs 32.7%).

In mice, Formoso-Rafferty et al. (2015) performed a divergent selection experiment for birth weight environmental variability. After seven generations, the high line had higher variance, standard deviation, and coefficient of variation than the low line (differences between lines were 167%, 59%, and 43%, respectively). Also, the birth weight was higher in the high line (1.64 g) than in the low line (1.47 g). This selection criterion affected other interesting traits such as litter size and survival at birth and weaning. Indeed, the low line showed a higher litter size at birth than the high line (9.77 kits vs 7.26 kits). In addition to a better survival at birth (98.47% in the low line vs 88.86% in the high line) and weaning (96.37% in the low line vs 79.64% in the high line) in the last generation of selection (Formoso-Rafferty et al., 2020).

In Pigs, Larzul et al. (2006) conducted a divergent selection experiment based on the homogeneity of ultimate pH. The standard deviation of this trait was lower in the homogeneous line than in the heterogeneous line for generations 2 and 3, but in the next generations, the experiment did not obtain good results, the authors did not considerate the experiment to be conclusive.

All of these previous studies have based on an indirect selection using the V_E genetic determination model described by SanCristobal-Gaudy et al. (1998). However, this model is highly complex and not robust (Yang et al., 2011). In order to avoid the use of complex models, Blasco et al. (2017) have made a simple and direct selection, i.e. by selecting for

environmental variance as an observed trait. This method seems to be more efficient, but its use is limited for cases of repeated measurements.

1.3. The relation between selection for environmental variation and animal welfare

According to the World Organisation from Animal Health (2014): “an animal is considered in a good state of welfare if it is healthy, comfortable, well-nourished, safe, able to express innate behaviour, and if it is not suffering from unpleasant states such as pain, fear, and distress”. Argente et al. (2019) have shownthat there is a close relation between environmental variability and animal welfare. Indeed, selection to reduce environmental variance would promote animal welfare by producing animals that cope better with their environment (Broom, 2008).

Welfare is a broad concept that has been related to such as robustness, canalisation, plasticity, and resilience (Colditz and Hine, 2016). All these traits are related to environmental variability or sensitivity to environmental perturbances.

Regarding robustness, there is no universally accepted definition. According to Knap (2005), robustness is the ability to combine some production potential with the capacity to endure external stressors, which allows expression in a wide variety of environmental conditions without compromising reproduction, health, and wellbeing. Bodin et al. (2010b) propose to improve robustness through increasing the tolerance to stress, and in consequence, decreasing sensitivity to variation in environmental factors.

Canalisation is defined as the reduction of phenotypic variability. Bodin et al. (2010b) defined canalisation referring only to the environmental variance instead of the whole phenotypic variance.

Phenotypic plasticity has been defined as the capacity of a genotype to produce different phenotypes most suitable depending on the external conditions experienced. Plasticity is about the environmental regulation of phenotype expression. Thus, plasticity plays a role in coping with environmental heterogeneity by allowing organisms to produce phenotypes adjusted to conditions that individuals will experience (Lafuente and Beldade, 2019).

Resilience is a concept with great interest in the last years (Scheffer et al., 2015), due to its links with welfare. Colditz and Hine (2016) defined resilience as the capacity of the animal to

cope with perturbations and rapidly return to the physiological, behavioural, health, and production states pertained before exposure to disturbance. Resilience is genetically related to environmental sensitivity (Mulder and Rashidi, 2017), thus resilience may be improved by genetic selection (Argente et al., 2019; Berghof et al., 2019).

2. Part 2

2.1. Microbiota

2.1.1. The gut microbiota

The body of animals is inhabited by a large number of microorganisms, such as bacteria, archaea, viruses, and eukaryotes, also known as microbiota (Kostic et al., 2013). The bacteria represent the principal community in the microbiota, for example in adult rabbits is about 80-90% (Carabaño et al., 2010). Due to the different physical conditions of temperature, gaseous condition and pH among internal cavities of the animal, we can differentiate an oral microbiota (Schueller et al., 2017), a stomach microbiota (Wu et al., 2014), a rumen microbiota for ruminants (Abecia et al., 2013), a vaginal microbiota for females (Kim et al., 2009) and a gut microbiota (Robles Alonso and Guarner, 2013).

The gut microbiota is considered the most important community, since it is the organ most strongly colonized with more than 70% of all the microbes from the mammalian body (Ley et al., 2006; Sekirov et al., 2010). The density and the composition of the microbiota vary throughout the gastrointestinal tract (Ouwehand and Vesterlund, 2003). Concerning microbial density, the stomachand the small intestine of rabbits show a lower quantity of microorganisms (10^4 to 10^6 and 10^5 to 10^8 bacteria /g of contents, receptively) than caecum (10^{11} to 10^{12} bacteria / g of contents), and feces (10^{10} to 10^{11} bacteria / g of contents) (Carabaño et al., 2006). In relation to microbial composition, the stomach and the small intestine of rabbits show a similar composition, being the Firmicutes the most abundant phyla (45.5%) followed by Bacteroidetes (20-30%), Proteobacteria (3.5%) and Actinobacteria (0.7%) (Crowley et al., 2017). In the distal segment of tract digestive as caecum and hard feces, Firmicutes increases its presence (76.54% in caecum and 76.28% in feces), followed by Tenericutes (7.83% in caecum and 8.17% in feces) and Verrucomicrobia (1.81% in caecum and 1.65% in feces), but Bacteroidetes (7.46% in caecum and 7.37% in feces) and

Proteobacteria (1.61% in caecum and 1.63% in feces) decrease its presence (Velasco-Galilea et al., 2018).

Most studies in rabbits have been focused on the microbiota of the caecum and hard feces, given the role of them in different metabolic and physiological processes in the host organism, as well as its influence on health and diseases. However, little is known about the microbial composition of the soft feces despite that could be similar to the caecal one. That would be an advantage for the study of the caecal microbiota since it would avoid the sacrifice of the animal.

The composition of the gut microbiota is affected by host-related factors such as age, diet composition, and the genome of host, among others.

Combes et al. (2014) found in 14-day-old lactating rabbits that Bacteroidetes are the most abundant phyla in the caecum with (63.3%), followed by Firmicutes (29.0%), Proteobacteria (6.9%) and Actinobacteria (0.7%). At weaning with the ingestion of solid food, this composition changes, and Firmicutes become the principal phyla (65.3%), followed by Bacteroidetes (32.9%), Proteobacteria (1.3%) and Actinobacteria (0.4%).

Food is an essential factor influencing the balance of microbial populations in the digestive tract, since it constitutes a substrate for organisms (Combes et al., 2011). The nature and quantity of food ingested influence the caecal ecosystem in rabbits (Gidenne et al., 2008). For example, a high-fiber diet has been related to an increasing both the diversity of the caecal microbiota (Michelland et al., 2011) and the ratio Firmicutes/Bacteroidetes (Combes et al., 2011), an indicator for microbiota maturity. On the other hand, it has been found that a diet with low energy and protein levels allows the microbial community to reach maturity faster (Read et al., 2019).

Concerning the genetics of the host, recent studies show that microbiota composition may also be determined by the genome of the individual where the microbiota is hosted (Blekhman et al., 2015; Bonder et al., 2016). Several studies have been carried out on the establishment of bacterial communities in twins and have concluded that there is a closer proximity of the dominant bacterial profiles in twins (Reyes et al., 2010; Lepage et al., 2011). In addition, mutations and the inactivation of certain specific genes have been associated with changes in the composition of the microbiota, sometimes linked to metabolic diseases (Spor et al., 2011).

2.1.2. Interaction between gut microbiota and immune system

The gut microbiota plays an important role in the development of the immune system and the activation of hypothalamic–pituitary–adrenal (HPA) axis, through releases a large amount of metabolites to the gut lumen and epithelial surface, such as short-chain fatty acids, vitamins, tryptophan, histamine, and polyamines (Bienenstock et al., 2015; Dodd et al., 2017; Kraimi et al., 2019; De Vadder et al., 2014; Masaki and Yoshimatsu, 2006; Pegg, 2013; Smith et al., 2013). Therefore, it is considered that the gut microbiota has a central role on animal welfare.

Concerning immunological functions on the host, it is well known that the gut microbiota is key in the formation of the barrier against pathogens (Collins et al., 2012). Indeed, the microbiota has an essential role in the development of the mucosa, which is an important surface of contact with external antigens (Sekirov et al., 2010). Besides, the gut microbiota is involved in the humoral immune response and the production of antibodies by stimulating the activation and the maturity of B cells (Petta et al., 2018). For instance, germ-free mice show a lower production of mature lymphocytes (Bouskra et al., 2008), and a significantly reduced immunoglobulin A (IgA) concentrations in the intestinal lumen (Macpherson and Harris, 2004), as well as fewer regulatory T cells (T_{regs}), which modulate the immune system, maintain tolerance to self-antigens and prevent autoimmune disease (Ostman et al., 2006). Thus, germ-free mice are more susceptible to infections and the development of diseases (Round and Mazmanian, 2009).

Besides, the metabolites produced by the gut microbiota also seem to have an important role in the host's immune response, through their immune-modulatory effect. For example, butyrate which is mainly secreted by the phyla of Firmicutes has direct effects on the immune system by regulating the T cells (Kunze and Hottiger, 2019). Acetate and propionate secreted by Bacteroidetes promote antibody production (Kim et al., 2016), production of anti-inflammatory cytokines IL-18 (Masaki and Yoshimatsu, 2006) and stimulate the expansion of pre-existing colonic T_{reg} cells (Arpaia et al., 2013).

In addition to microbial metabolites, certain components of the bacterial cell wall, such as lipopolysaccharides and peptide glycans, are also acted on innate immune response through activation of the Toll-like receptors and macrophages (Kamada et al., 2013). Toll-like receptors participate in the activation of transcription factors NF-kB and AP1, which regulate the expression of inflammatory cytokines such as TNFα, IL1, and IL6 (Moynagh, 2005).

Also, macrophages activate the production of cytokines (Zhang and An, 2007). Cytokines play an important role in the innate immune response through direct mechanisms against the invading agent (inhibiting viral replication). Besides, cytokines have specific effects on interactions and communications between cells (Zhang and An., 2007). Table 2 illustrates some cytokines, their sources and their possible activities on the immune system.

Table 2. Cytokines and their immune activities (Zhang and An, 2007).

Cytokines	Sources	Activities
IL1-α and IL1-β	Macrophages and other antigen presenting cells (APCs)	Costimulation of APCs and T lymphocytes, inflammation, fever, acute phase response
IL6	Activated Th2 cells and APCs, other stomatal cells	Acute phase response, proliferation of B lymphocytes, in synergy with IL1and TNF on T lymphocytes
IL2	Th1 activated lymphocytes	Proliferation of B and T lymphocytes
TNF-α	T cells, monocytes, macrophages, mast cells, sensory neurons	Chemotaxis, synthesis of IL1 and IL6, cell death, inflammation, pain

IL1-α : Interleukin 1 alpha; IL1-β: Interleukin 1 beta; IL1: Interleukin 1; IL6: Interleukin 6; IL2: Interleukin 2; TNF-α: Tumor Necrosis Factor alpha; APCs: Antigen Presenting Cells; Th1: T helper1; Th2: T helper 2.

In conclusion, the gut microbiota participates in the development and activation of the immune system through the release of different metabolitesas short-chain fatty acids, vitamins, tryptophan, histamine, and polyamines.

2.2. Metagenomics

Metagenomics analyses all the genomes present in an ecosystem (Streit and Schmitz, 2004; Langer et al., 2006), and has become a powerful tool for analysing microbial communities both in terms of what species are present and the activity of which they are capable to carry out, without the need to isolate the microorganisms. Therefore, the metagenomics approach is a major advance in exploring the different microbial ecosystems, such as the gastrointestinal flora of farm animals.

- **Using 16S RNA gene sequencing**

The 16S Ribosomal RNA gene (16S) is a marker of bacteria and archaea frequently used to characterize the taxonomic composition and phylogenetic diversity (Langille et al., 2013). The 16S sequences are grouped into operational taxonomic units (OTUs). An OTU is therefore a group of bacteria belonging to the same species whose 16S sequences have a similarity of more than 97.5%. This approach consists in cloning the genes coding for the 16S rRNA, which were then sequenced by the Sanger method (Martin, 1989) with a cost significantly less expensive than the next-generation technique. The 16S makes possible to identify the most organisms within a community, but the analysis of less abundant organisms is limited or in the case of virus, fungi, and protozoa cannot be detected. Moreover, this technique does not provide any other information about the genomes of its members or their function.

- **Next-generation sequencing (Shotgun Metagenomics)**

In recent years, the rapid development of next-generation sequencing techniques has allowed a large number of taxa to be sequenced much more efficiently than with Sanger-type sequencing. Moreover, this technique avoids the previous step of cloning, which is necessary for the 16S rRNA technique, with its associated bias, allowing microbial communities to be investigated with higher resolution and identifying less abundant taxa (Maccaferri et al., 2011). Besides, metagenomics provides access to the functional gene composition of

microbial communities and thus gives a much broader description than phylogenetic surveys (Thomas et al., 2012).

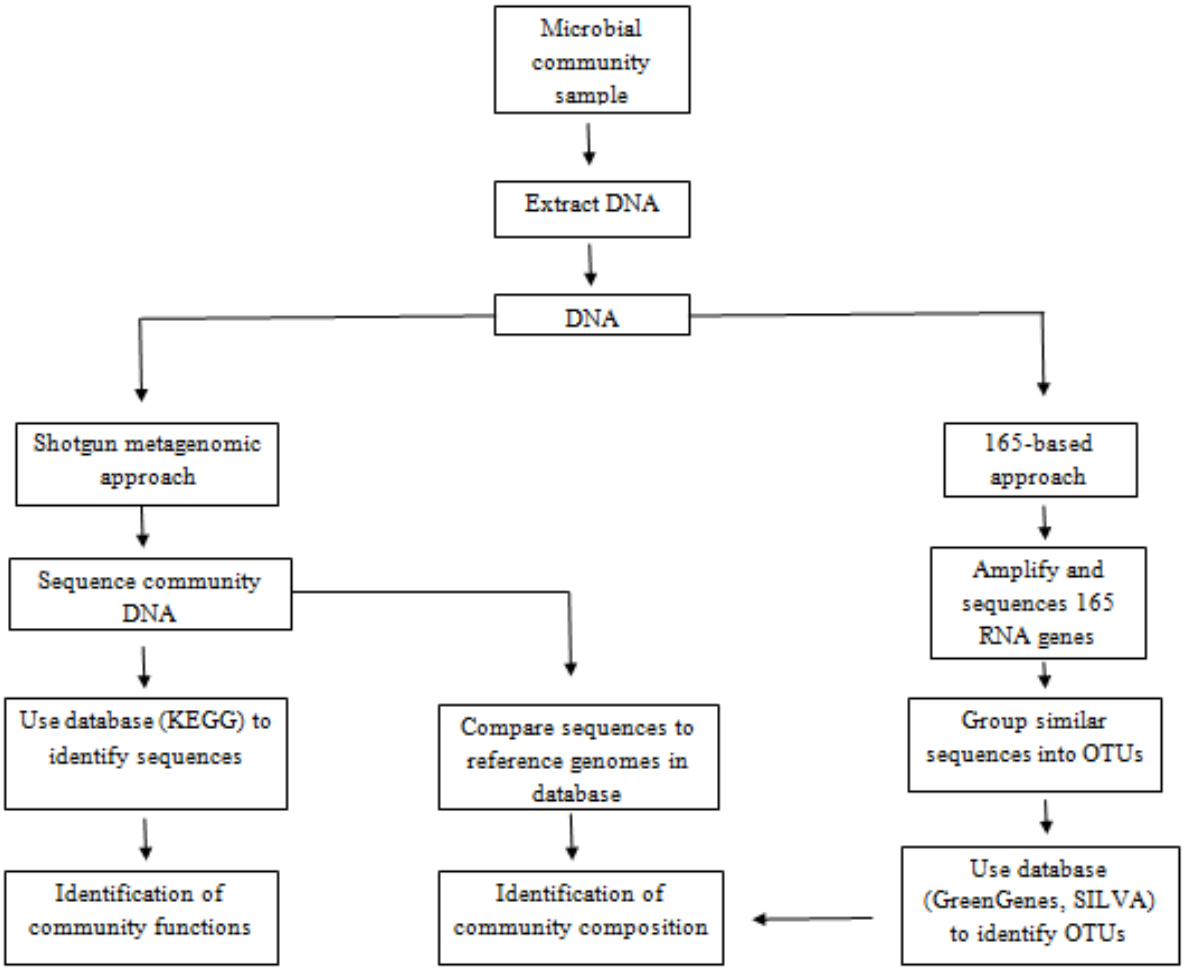

Figure 1. Schematic comparison of the two most widely used approaches: 16S rRNA and Shotgun Metagenomics (from Wilson, 2018).

The development of bioinformatic methods for metagenomics has focused on two questions, which are the taxonomic and functional characterization of microbial communities (Figure 1).

- **Taxonomic characterisation**

The taxonomic characterisationis traditionally used. It consists of comparing the metagenomic sequences to sequences representative of each taxon, like their reference genome. Taxonomic characterization mainly tends to classify the different microorganisms inhabiting a given

environment (Awasthi et al., 2020). The classification is from higher taxonomic levels (e.g. superkingdom or phylum) until lower taxonomic levels (e.g. genus or species).

- **Functional metagenomics**

If the microbiota constitutes half of a holobiont in mammals in the number of cells (that is approximately 3.9 x 10^{13} bacteria), the number of microbial genes compared to the number of human genes is 100 times greater (Sender et al., 2016). Gene prediction identifies the coding sequences for a protein in the contigs. Functional annotation is the process that collects information about gene's biological identity and associates each gene with a function (Berardini et al., 2004). Finally, all functions detected in a metagenome can be linked to reconstruct metabolic pathways (Guyomar, 2018). Functional annotation is done using databases such as KEGG.

The KEGG database is a set of databases grouping together different types of biological data and provides annotation at different levels (Kanehisa et al., 2004). It provides four main databases: GENES for genomic information, PATHWAY for pathways of biological processes, LIGAND for chemical compounds and reactions, and BRITE for ortholog/paralog information as well as other classification systems (Aoki-Kinoshita and Kanehisa, 2007).

REFERENCES

Abecia L, Martín-García AI, Martínez G, Newbold CJ and Yáñez-Ruiz DR 2013. Nutritional Intervention in Early Life to Manipulate Rumen Microbial Colonization and Methane Output by Kid Goats postweaning1. Journal of Animal Science 91, 4832–40.

Agea I, García ML, Blasco A and Argente MJ 2019. Litter Survival Differences between Divergently Selected Lines for Environmental Sensitivity in Rabbits. Animals 9: 603.

Aoki-Kinoshita KF and Kanehisa M 2007. Gene Annotation and Pathway Mapping in KEGG. In: Bergman N.H. (eds) Comparative Genomics. Methods In Molecular Biology™, 396. Humana Press.

Arpaia N, Campbell C, Fan X, Dikiy S, Van JDV, Deroos P, Liu H, Cross JR, Pfeffer K and Coffer PJ 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 504: 451

Argente MJ, Calle EW, García ML and Blasco A 2017. Correlated response in litter size components in rabbits selected for litter size variability. Journal of Animal Breeding and Genetics 134, 505-511.

Argente MJ, García ML, Zbyňovká K, Petruška P, Capcarová M and Blasco A 2019. Correlated response to selection for litter size environmental variability in rabbit's resilience. Animal 13, 2348-2355.

Awasthi MK, Ravindran B, Sarsaiya S, Chen H, Wainaina S, Singh E, Tao L, Kumar S, Pandey A, Singh L and Zhang Z 2020. Metagenomics for taxonomy profiling: tools and approaches. Bioengineered 11, 356-374.

Berardini TZ, Mundodi S, Reiser L, Huala E, Garcia-Hernandez M,…and Rhue SY 2004. Funcional Annotation of the Arabidopsis Genome Using Controlled Vocabularies. Plant Physiol 135, 745-755.

Berghof TVL, Poppe M and Mulder HA 2019. Opportunities to improve resilience in animal breeding programs. Front. Genet. 9: 692.

Bienenstock J, Kunze W and Forsythe P 2015. Microbiota and the gut-brain axis. Nutri. Rev 73, 28-31.

Blasco A, Martínez-Álvaro M, García ML, Ibáñez-Escriche N and Argente MJ 2017. Selection for environmental variance of litter size in rabbits. Genet Sel Evol 49, 48-55.

Blekhman R, Goodrich JK, Huang K, Sun Q, Bukowski R, Bell JT, Spector TD, Keinan A, Ley RE, Gevers D and Clark AG 2015. Host genetic variation impacts microbiome composition across human body sites. Genome Biology 16: 191.

Bodin L, Garcia M, Saleil G, Bolet G and Garreau H 2010a. Results of 10 generations of canalising selection for rabbit birth weight. Results of canalizing selection for rabbit birth weight. Proc. 9thWCGALP, Liepzig, Germany, 2-6 august 2010.

Bodin L, Bolet G, Garcia M, Garreau H, Larzul C and David I 2010b. Robustesse et canalisation: vision de généticiens. INRA Prod. Anim 23, 11-22.

Bonder MJ, Kurilshikov A, Tigchelaar EF, Mujagic Z, Imhann F, ... and Zhernakova DV 2016. The effect of host genetics on the gut microbiome. Nature genetics 48, 1407-1412.

Bouskra D, Brezillon C, Berard M, Werts C, Varona R, Boneca IG and Eberl G 2008. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature 456, 507-510.

Broom DM 2008. Welfare assessment and relevant ethical decisions: key concepts. Ann. Rev. Biomed. Sci 20, 79-90.

Calle EW, García ML, Blasco A and Argente MJ 2017. Correlated response in early embryonic development in rabbits selected for litter size variability. World Rabbit Science 25, 323-327.

Carabaño R, Badiola I, Licois D and Gidenne T 2006. The digestive ecosystem and its control through nutritional or feeding strategies. Recent advances in rabbit sciences, 211-227.

Carabaño R, Piquer J, Menoyo D and Badiola I 2010. The digestive system of the rabbit. CAB International 2010. Nutrition of the rabbit, 2nd Edition.

Colditz I and Hine B 2016. Resilience in farm animals: Biology, management, breeding and implications for animal welfare. Animal Production Science 56, 1961-1983.

Collins SM, Surette M and Bercik P 2012. The interplay between the intestinal microbiota and the brain. Nature reviews Microbiology 10, 735-42.

Combes S, Fortun-Lamothe L, Cauquil L and Gidenne T 2011. Piloter l'écosystème digestif du lapin : pourquoi, quand et comment. 14ème Journées de la Recherche Cunicole, 22-23 novembre 2011, Le Mans, France.

Combes S, Gidenne T, Cauquil L, Bouchez O and Fortun-Lamothe L 2014. Coprophagous behavior of rabbit pups affects implantation of cecal microbiota and health status. J. Anim. Sci 92, 652-665.

Crowley EJ, King JM, Wilkinson T, Worgan HJ, Huson KM, Rose MT and McEwan NR 2017. Comparison of the microbial population in rabbits and guinea pigs by next generation sequencing. PLoS One 12: e0165779.

De Vadder F, Kovatcheva-Datchary P, Goncalves D, Vinera J, Zitoun C, Duchampt D, Backhed F and Mithieux G 2014. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. Cell156, 84-96.

Dodd D, Spitzer MH, Van Treuren W, Merrill BD, Hryckowian AJ, Higginbottom SK, Le A, Cowan TM, Nolan GP, Fischbach MA and Sonnenburg JL 2017. A gut bacterial pathway metabolizes aromatic amino acids into nine circulating metabolites. Nature 551, 648-652.

Falconer D S and Mackay TCF 1996. Introduction to Quantitative Genetics (London, Longman).

Fina M, Ibañez-Escriche N, Piedrafita J and Casellas J 2013. Canalization analysis of birth weight in Bruna delsPirineus beef cattle. J Anim Sci 91, 3070-3078.

Formoso-Rafferty N, Cervantes I, Ibañez-Escriche N and Gutiérrez JP 2015. Genetic control of the environmental variance for birth weight in seven generations of a divergent selection experiment in mice. Journal of Animal Breeding and Genetics, doi: 10.1111/jbg.12174.

Formoso-Rafferty N, Cervantes I, Ibañez-Escriche N and Gutiérrez JP 2016. Correlated genetic trends for production and welfare traits in a mouse population divergently selected for birth weight environmental variability. Animal 10, 1770-1777.

Formoso-Rafferty N, Chavez KN, Ojeda C, Cervantes I and Gutiérrez JP 2020. Selection Response in a Divergent Selection Experiment for Birth Weight Variability in Mice Compared with a Control Line. Animals 10: 920.

Garreau H, Bolet G, Larzul C, Rovert-Granié C, Saleil G, SanCristobal M and Bodin L 2008. Results of four generations of a canalising selection for rabbit birth weight. Livest. Sci 119, 55-62.

Gidenne T, Combes S, Licois D, Carabaño R, Badiola I and Garcia J 2008. Ecosystème caecal et nutrition du lapin : interactions avec la santé digestive. INRA Prod. Anim 21, 239-250.

Gutiérrez JP, Nieto B, Piqueras P, Ibáñez N, Salgado C 2006. Genetic parameters for canalisation analysis of litter size and litter weight traits at birth in mice. Genetics Selection Evolution 38, 445-462.

Guyomar C 2018. Développement et applications d'outils d'analyse métagénomique des communautés microbiennes associées aux insectes. Doctoral thesis. Sciences agricoles. Université Rennes 1. NNT: 2018REN1B049f.

Hill WG and Mulder HA 2010. Genetic analysis of environmental variation. Genet.Res.Camb 92, 381-395.

Ibáñez-Escriche N, Varona L, Sorensen D and Noguera JL 2008a. A study of heterogeneity of environmental variance for slaughter weight in pigs. Animal 2, 19-26.

Ibáñez-Escriche N, Moreno A, Nieto B, Piqueras P, Salgado C, Gutiérrez JP 2008b. Genetic parameters related to environmental variability of weight traits in a selection experiment for weight gain in mice; signs of correlated canalised response. Genetics Selection Evolution 40, 279-293.

Kamada N, Chen GY, Inohara N and Nuñez G 2013. Control of pathogens and pathobionts by the gut microbiota. Nat. Immunol 14, 685-690.

Kanehisa M, Goto S, Kawashima S, Okuno Y and Hattori M 2004. The kegg resource for deciphering the genome. Nucleic acids research 32, 277-280.

Kim M, Qie Y, Park J and Chang HK 2016. Gut microbial metabolites fuel host antibody responses. Cell Host Microbe 20, 202-214.

Kim TK, Thomas SM, Ho M, Sharma S, ... and Wilson BA 2009. "Heterogeneity of Vaginal Microbial Communities within Individuals". Journal of Clinical Microbiology 47, 1181-1189.

Knap PW 2005. Breeding robust pigs. Australian Journal of Experimental Agriculture 45, 763-773.

Kostic AD, Howitt MR and Garrett WS 2013. Exploring host–microbiota interactions in animal models and humans. Genes Dev 27, 701-718.

Kraimi N, Dawkins M, Gebhardt-Henrich SG, Velge P, Rychlik I, Volf J, Creach P, Smith A, Colles F and Leterrier C 2019. Influence of the microbiota-gut-brain axis on behavior and welfare in farm animals: A review. Physiology & Behavior 210: 112658.

Kunze FA and Hottiger MO 2019. Regulating Immunity via ADP-Ribosylation: Therapeutic Implications and Beyond. Trends in Immunology 40, 159-173.

Lafuente E and Beldade P 2019. Genomics of Developmental Plasticity in Animals. Front Genet 10: 720.

Langer L, Gabor E, Liebeton K, Meurer G, Niehaus F, Schulze R, Eck J and Lorenz P 2006. Metagenomics: An inexhaustible access to nature's diversity. Biotechnology Journal 1, 815-821.

Langille MGI, Zaneveld J, Caporaso G, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Thurber RLV, Rob Knight R, Robert G, Beiko RG and Huttenhower C 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 31, 814-821.

Larzul C, Le Roy P, Tribout T, Gogue J and SanCristobal M 2006. Canalyzing selection on ultimate pH in pigs: consequences on meat quality. Porc 8th WCGALP, August 13-18, Belo Horizonte, Brasil, 4p.

Lepage P, Hasler R, Spehlmann ME, Rehman A, Zvirbliene A, Begun A, Ott S, Kupcinskas L, Doré J, Raedler A and Schreiber S 2011. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. Gastroenterology 141, 227-236.

Ley RE, Peterson DA and Gordon JI 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124, 837–848.

Maccaferri S, Biagi E and Brigidi P 2011. Metagenomics: key to human gut microbiota. Dig Dis 29, 525-30.

Martin WJ 1989. New technologies for large-genome sequencing. Genome 31, 1073-1080.

Masaki T and Yoshimatsu H 2006. The hypothalamic H1 receptor: a novel therapeutic target for disrupting diurnal feeding rhythm and obesity. Trends Pharmacol Sci 27, 279-284.

Michelland RJ, Combes S, Monteils V, Cauquil L, Gidenne T and Fortun-Lamothe L 2011. Rapid adaptation of the bacterial community in the growing rabbit caecum after a change in dietary fibre supply. Animal 5, 1761-1768.

Moynagh PN 2005. TLR signalling and activation of IRFs: revisiting old friends from the NFkappaB pathway. Trends Immunol 26, 469-476.

Mulder HA, Hill WG, Vereijken A and Veerkamp RF 2009. Estimation of genetic variation in residual variance in female and male broilers. Animal 3, 1673-1680.

Mulder HA, Hill WG and Knol EF 2015. Heritable environmental variance causes nonlinear relationships between traits: application to birth weight and stillbirth of pigs. Genetics 199, 1255-1269.

Mulder HA and Rashidi H 2017. Selection on resilience improves disease resistance and tolerance to infections. Journal of Animal Science 95, 3346-3358.

Ostman S, Rask C, Wold AE, Hultkrantz S and Telemo E 2006. Impaired regulatory T cell function in germ-free mice. Eur J Immunol 36, 2336-2346.

Ouwehand A and Vesterlund S 2003. Health aspects of probiotics. IDrugs 6, 573-580.

Pegg AE 2013. Toxicity of polyamines and their metabolic products. Chem Res Toxicol 26, 1782-1800.

Petta I, Fraussen J, Somers V and Kleinewietfeld M 2018. Interrelation of Diet, Gut Microbiome, and Autoantibody Production. Front Immunol 9: 439.

Read T, Fortun-Lamothe L, Pascal G, Le Boulch M, Cauquil L, Gabinaud B, Bannelier C, Balmisse E, Destombes N, Bouchez O, Gidenne T and Combes S 2019. Diversity and Co-occurrence Pattern Analysis of Cecal Microbiota Establishment at the Onset of Solid Feeding in Young Rabbits. Front Microbiol 10: 973.

Reyes A, Haynes M, Hanson N, Angly FE, Heath AC, Rohwer F and Gordon JI 2010. Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature 466, 334-338.

Robles Alonso V and Guarner F 2013. Linking the Gut Microbiota to Human Health. Br J Nutr 109, 21-26.

Rönnegård L, Felleki M, Fikse WF, Mulder H and Strandberg E 2013. Variance component and breeding value estimation for genetic heterogeneity of residual variance in Swedish Holstein dairy cattle. Journal of Dairy Science 96, 2627-2636.

Round JL and Mazmanian SK 2009. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 9, 313-323.

SanCristobal-Gaudy M, Elsen JM, Bodin L and Chevalet C 1998. Prediction of the response to a selection for canalisation of a continuous trait in animal breeding. Genetics Selection Evolution 30, 423-451.

Scheffer M, Carpenter SR, Dakos V and Van Nes EH 2015. Generic indicators of ecological resilience: inferring the chance of a critical transition. Annu. Rev. Ecol. Evol. Syst 46, 145-167.

Schueller, K, Riva A, Pfeiffer S, Berry D and Somoza V 2017. Members of the Oral Microbiota Are Associated with IL-8 Release by Gingival Epithelial Cells in Healthy Individuals. Frontiers in Microbiology 8, 416-425.

Sekirov I, Russell SL, Antunes LC and Finlay BB 2010. Gut microbiota in health and disease. Physiol Rev 90, 859-904.

Sell-Kubiak E, Bijma P, Knol EF and Mulder HA 2015. Comparison of methods to studyuniformity of traits: application to birth weight in pigs. Journal of Animal Science 93, 900-911.

Sender R, Fuchs S and Milo R 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLOS Biology 14:e1002533.

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN and Garrett WS 2013. The microbial metabolites, shortchain fatty acids, regulate colonic Treg cell homeostasis. Science 341, 569-573.

Sorensen D and Waagepetersen R 2003. Normal linear models with genetically structured residual variance: a case study. Genetics Research 82, 202-222.

Spor A, Koren O and Ley R 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. Nature Rev. Microbiol 9, 279-290.

Streit W and Schmitz R 2004. Metagenomics – the key to the uncultured microbes. Current Opinion in Microbiology 7, 492-498.

Thomas T, Gilbert J and Meyer F 2012. Metagenomics - a guide from sampling to data analysis. Microb Inform Exp 2: 3.

Velasco-Galilea M, Piles M, Viñas M, Rafel O, González-Rodríguez O, Guivernau M and Sánchez JP 2018. Rabbit Microbiota Changes Throughout the Intestinal Tract. Front Microbiol 9, 2144-2157.

Wilson M 2018. The Human Microbiota in Health and Disease: An Ecological and Community. Garland Science 505.

World Organisation for Animal Health 2014. Terrestrial Animal Health Code. Chapter 7.1. Availableat http://www.oie.int/en/international-standard-setting/terrestrial-code/access online/.

Wu WM, Yang YS and Peng LH 2014. Microbiota in the Stomach: New Insights. Journal of Digestive Diseases 15, 54-61.

Yang Y, Christensen OF and Sorensen D 2011. Analysis of a genetically structured varianceheterogeneity model using the Box-Cox transformation. Genetics Research 93, 33-46.

Zhang JM, and An J 2007. Cytokines, inflammation, and pain. Int. Anesthesiol. Clin 45, 27-37.

Chapter 2. Objectives

The main objectives of this study were:

- To analyse the inflammatory response in the high and the low line in order to study the effect of selection for the environmental variability of litter size on susceptibility to diseases.
- To analyse the existence of microbial genes associated with the environmental variability of litter size that allows us to separate the high and the low line.

Chapter 3. Inflammatory correlated response in two lines of rabbit selected divergently for litter size environmental variability

ABSTRACT

A divergent selection experiment for environmental variance of litter size variance was carried out in rabbits during thirteen generations. The aim of this study was to analyse the inflammatory response in the high and the low line in order to study the effect of selection on susceptibility to diseases. A total of 78 females were used in this study, 39 from each line. The line selected for litter size heterogeneity (the high line) showed lower white blood leukocyte count (WBC) (-0.87 $x10^3$/µl), lower percentage of basophils (-0.11%), higher concentration of TNF-α (+13.8 pg/ml) and greater concentration of CRP (+38.1µg/ml) than the line selected for litter size homogeneity (the low line). The high line had also higher concentrations of bilirubin, cholesterol, gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) compared to the low line (difference between lines +0.08 µmol/L, +0.14 µmol/L, +0.35 U/L and +2.4 U/L and, respectively). The high line showed higher inflammatory response and susceptibility to infectious disorders. In conclusion, the line selected to increase litter size environmental variability seems to have a poor capacity to cope with environmental stressors.

Keywords: C-reactive protein, divergent selection response, rabbits, susceptibility to diseases, welfare.

INTRODUCTION

In the last few decades, animal welfare has become a priority objective for farmers and the livestock industry (Bodin et al., 2010b). Animal welfare is defined as the capacity of animals to cope with their environment (Broom, 2008). Susceptibility to stress and diseases are closely related to this adaptation (Mormede et al., 2018), playing an important role for the immune system in this process (Scrivo et al., 2011). Inflammation is the immune system's response that is triggered to microbial invasion or tissue damage in order to maintain the body's homeostasis (Liu et al., 2017). Inflammation is a complex process which involves a high number of molecules (Del Giudice and Gangestad, 2018). The cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) together the acute-phase protein C-reactive protein (CRP) are main used inflammatory biomarkers. Levels of these biomarkers help to detect the presence and severity of inflammation (Lockwood et al., 2017).
In prolific species such as pigs, rabbits and mice, environmental variability in body weight and in litter size has been related to immune response and resistance to diseases (see review

by Lung et al., 2020). A divergent selection experiment for environmental variance of litter size at birth was performed in rabbits. After ten generations of selection, the lines showed a remarkable divergent response (2.7 kits^2 in the low line vs. 4.4 kits^2 in the high one) (Blasco et al., 2017). Besides, females selected for litter size homogeneity (the low line) showed lower levels of cortisol and CRP than those selected for litter size heterogeneity (the high line) (Argente et al., 2019). This would agree with a better cope to environmental stressors, including chronic stressors, inflammation, and diseases in the homogenous line. Liver has an important role in the synthesis of acute-phase proteins, as CRP, in response to cytokines such as IL-6, TNF-α and IL-1β (Stoner et al., 2013). Moreover, liver metabolism has been related to reduce the inflammatory process (Otero et al., 2009).

The objective of this study was to analyse the inflammatory response in the two lines of the divergent selection experiment for litter size environmental variability, in order to analyse the effect of selection on susceptibility to diseases. For this purpose, additional inflammatory and biochemical markers to the ones studied by Argente et al. (2019) were measured in the 13th generation of the experiment.

MATERIAL AND METHODS

Ethics statement

All experimental procedures were approved by the Miguel Hernández University of Elche Research Ethics Committee, according to Council Directives 98/58/EC and 2010/63/EU (reference number 2017/VSC/PEA/00212).

Experiment animals

A divergent selection experiment on environmental variability of litter size was carried out during thirteen generations. Selection was based on the phenotypic variance of litter size of each doe, after correcting litter size for both year-season and parity-lactation status (first parity, and lactating or not at mating in other parities) (Blasco et al., 2017). A total of seventy-eight primiparous female rabbits from the 13th generation, 39 from the line selected for litter size heterogeneity (the high line) and 39 from the line selected for litter size homogeneity (the low line) were used in this experiment. All the animals were reared in the farm of the Miguel Hernández University of Elche (Spain). The rabbits were fed a standard commercial diet (17% crude protein, 16% fiber, 3.5% fat, NutricunElite Gra®, De HeusNutrición Animal, La Coruña, Spain). Food and water were provided ad libitum. Does were housed in individual

cages (37.5 cm x 33 cm x 90 cm) under a constant photoperiod of 16 h continuous light: 8 h continuous darkness, and with controlled ventilation. The experimenttook place from March to July. The temperature ranged from 15.2ºC to 32.1ºC. Reproduction was organized in discrete generations. All does were mated at the same age, i.e. at 18 weeks of age.

Table 3. Distribution of number of does (n) and live weight per month.

	n	Live weight (g)
March	17	3395
April	16	3480
May	16	3290
June	19	3440
July	10	3475

Blood collection

Following the blood-sampling procedure described in Lidforsand Edström (2013), two blood samples of 3 ml were drawn from the central artery of each doe's ear 24 hours after the first delivery at twenty-two weeks of age. Delivery is a stressful event to does which may have an influence bearing on haematological and biochemical parameters. The first blood sample was collected into a tube with tripotassium ethylenediaminetetraacetic acid (K3-EDTA). This sample was divided into two aliquots. One aliquot was used for haematology, and the other one was centrifuged (at 4000 rpm for 15 min) in order to determine concentrations of C-reactive protein (CRP), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and cortisol. The plasma samples obtained by centrifugation were stored at -80ºC until further analysis. The second blood sample was collected into a lithium heparin tube, after centrifugation the concentrations of bilirubin, cholesterol, alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), albumin (ALB), bile acid (BA), and blood urea nitrogen (BUN) were assessed.

Haemogram

Haematological parameters such as white blood leukocyte count (WBC) and the percentage of lymphocytes, neutrophils, monocytes, basophils and eosinophils were done by the haematology analyser Abacus Junior Vet (Diatron, Austria).

Assessment of C-reactive protein, cytokines (interleukin 6 and tumor necrosis factor alpha) and cortisol

C-reactive protein (CRP) concentration was quantified using a commercially available ELISA kit for rabbits (catalogue number 2210-5; Life Diagnostics Inc., West Chester, PA, USA). Interleukine 6 (IL-6) concentration was analysed using a commercially available ELISA kit for rabbits (catalogue number CSB E06903Rb; Cusabio, USA). The concentration of tumor necrosis factor alpha (TNF-α) was quantified using a commercially available ELISA kit for rabbits (catalogue number EL RB0011; Elabscience, USA). Cortisol plasma concentration was performed using available ELISA kit for rabbits (catalogue number CSB E06956Rb; Cusabio, USA).

Assessment of biochemical parameters

Plasma concentrations of bilirubin, cholesterol, alkaline phosphatase, gamma glutamyl transferase, albumin, bile acid and blood urea nitrogen were evaluated using the VetScan® Mammalian Liver Profile Rotor from Abaxis Company.

Statistical Analysis

Data were analysed using the following model:

$$y_{ijk} = \mu + MS_i + L_j + b\, x_{ijk} + e_{ijk}$$

where MS_i is the month of blood sampling effect with five levels, L_j the line effect with two levels (high and low line), b is the regression coefficient, x_{ijk} is the covariate weight and e_{ijk} is the residual term. Residuals were assumed to be independently normally distributed with the same variance. A Bayesian analysis was used, with bounded flat priors for all unknown parameters. Marginal posterior distributions were estimated for all unknowns using Gibbs sampling. Marginal posterior distributions of the differences between lines were computed with the program Rabbit developed by the Institute for Animal Science and Technology (Valencia, Spain). Monte Carlo Markov chains of 60000 iterations, with a burn-in period of 10000, and only one out of every 10 samples was saved for inferences. Convergence was tested using the Z criterion of Geweke and Monte Carlo sampling errors were computed using time-series procedures. Bayesian statistic gives a new approach to the description of the uncertainly against classical statistics. For example, we can provide the difference between lines (D_{H-L}) and the precision of our estimation, finding the shortest interval with 95% probability of containing the true value that can be asymmetric around the estimation (this is called the highest posterior density interval at 95% probability). Notice that in Bayesian

context there is nothing like `significance' (Blasco, 2017), but we can calculate the actual probability of the difference between the high and low line $|D_{H-L}|$ being higher than zero; this is much more informative than P-values and significance, see Blasco. (2017) for details. We consider that there is enough evidence about the high and the low line being different, when the probability of this difference in absolute value $|D_{H-L}|$is more than 90%, however this is not a significance test, but is a way to help the discussion, since we have all actual probabilities of the differences between lines and the reader can consider other probability as being relevant enough to differentiate both lines.

RESULTS

Immune parameters

Table 4 shows the features of the estimated marginal posterior distributions of the differences between lines (D_{H-L}) for the haematological parameters. The high line had lower white blood leukocyte count (WBC) (-0.87 $x10^3$/µl, P = 0.95) and lower percentage of basophils than the low line (-0.11%, P = 0.93). We did not observe differences between lines for the percentages of lymphocytes, neutrophils, monocytes, and eosinophils.

Table 4. Immune parameters in female rabbits from the high and the low line.

	H	L	D_{H-L}	$HPD_{95\%}$	P
WBC (x103/µl)	7.48	8.35	-0.87	-1.8, 0.15	0.95
Lymphocytes (%)	60.2	61.9	-1.7	-10.4, 6.73	0.66
Neutrophils (%)	33.6	31.9	1.7	-6.52, 9.94	0.66
Monocytes (%)	3.5	3.16	0.34	-0.5, 1.2	0.77
Eosinophils (%)	2.28	2.41	-0.13	-0.67, 0.45	0.69
Basophils (%)	0.42	0.53	-0.11	-0.26, 0.03	0.93

H: median of the line selected for litter size heterogeneity (the high line); L: median of the line selected for litter size homogeneity (the low line); D_{H-L}: differences between the high line and the low line; $HPD_{95\%}$: highest posterior density region at 95%; P: probability of the difference being > 0 when $D_{H-L} > 0$ or being < 0 when $D_{H-L} < 0$; WBC: white blood cells.

Cytokines, C-reactive protein (CRP) and cortisol

According to Table 5, the high line showed higher concentration of TNF-α (+13.8 pg/ml, P = 0.90) and greater concentration of CRP in comparison with the low line (+38.1µg/ml, P =

1.00). Concentrations of Interleukin 6 (IL-6) and cortisol were similar in both lines (P = 0.55 and P = 0.60 respectively).

Table 5. Cytokines, C-reactive protein (CRP), and cortisol concentrations in female rabbits from the high and the low line.

	H	L	D_{H-L}	$HPD_{95\%}$	P
IL-6 (pg/ml)	84.8	85.2	-0.6	-8.6, 7.2	0.55
TNF-α (pg/ml)	50.1	36.3	13.8	-9.2, 36.6	0.90
CRP (µg/ml)	85.5	47.4	38.1	15.8, 60.8	1.00
Cortisol (ng/ml)	24.5	25.1	-0.6	-4.7, 3.5	0.60

H: median of the line selectedfor litter size heterogeneity (the high line); L: median of the line selected for litter size homogeneity (the low line); D_{H-L}: differences between the high line and the low line; $HPD_{95\%}$: highest posterior density region at 95%; P: probability of the difference being>0 when $D_{H-L}>0$ or being<0 when $D_{H-L}<0$; IL-6:interleukin 6; TNF-α: tumor necrosis factor-alpha.

Biochemical parameters

Features of the marginal posterior distributions of the differences between the high and the low lines for biochemical parameters are given in Table 6. The concentrations of bilirubin and GGT were higher in the high line than the low line P = 0.88, P = 0.89, respectively, cholesterol and ALP levels were also higher in the high line than in the low one (+0.14 µmol/L and +2.4 U/L, P > 0.90). There is some evidence of having differences between lines in BA (P = 0.84). No differences between the high and low lines were found for ALB and BUN (P = 0.61 and P = 0.54).

Table 6. Biochemical parameters concentrations in female rabbits from the high and the low line.

	H	L	D_{H-L}	HPD95%	P
Bilirubin (µmol/L)	4.74	4.66	0.08	-0.05, 0.2	0.88
Cholesterol (µmol/L)	1.24	1.1	0.14	-0.07, 0.34	0.91
ALP (U/L)	21.1	18.7	2.4	-0.92, 5.62	0.93
GGT (U/L)	5.98	5.63	0.35	-0.20, 0.92	0.89
BA (µmol/L)	3.15	2.59	0.56	-0.54, 1.68	0.84
ALB (g/L)	13.6	13.5	0.1	-0.77, 1.07	0.61
BUN (µmol/L)	7.56	7.62	-0.06	-1.06, 0.97	0.54

H: median of the line selected for litter size heterogeneity (the high line); L: median of the line selected for litter size homogeneity (the low line); D_{H-L}: differences between the high line and the low line; $HPD_{95\%}$: highest posterior density region at 95%; P: probability of the difference being > 0 when $D_{H-L} > 0$ or being < 0 when $D_{H-L} < 0$; ALP: alkaline phosphatase; GGT: gamma-glutamyl transferase; BA: bile acid; ALB: albumin; BUN: blood urea nitrogen.

DISCUSSION

Immune parameters

Haematological parameters provide valuable information on the health status of the animal. In the present study, we evaluated the haematological profile in the two lines at 24 hours after the first delivery. We found that there was no difference between lines, except for WBC and the percentage of basophils. The WBC values obtained were within the normal range for rabbits (2.71 -12.23 $x10^9$/L) reported by (Leineweber et al., 2018). Total leucocyte counts are involved in immunity reaction and defence of the organism (Mbanasor et al., 2003; Ihedioha et al., 2008). Susceptible rabbits to acute infections may have a decrease in WBC count (Moore et al., 2015). On the other hand, basophils are essential for linking innate and adaptive immunity (Ma and Gao, 2020). Therefore, a higher basal concentration of WBC and basophils in the low line would be related to a better disease resistance and a good immunity. Lymphocytes and neutrophils are important for the immune system. Neutrophils provide the first line defence against infection in innate immune response (Furze and Rankin, 2008). Lymphocytes are involved in humoral and cell-mediated immunity response (Mahgoub et al., 2008). In a previous study, Argente et al. (2019) found a higher basal concentration of neutrophils and lower concentration of lymphocytes in the low line. However, no differences between lines found in this study. We would like to note that sample collection was carried out at different environmental stress condition, i.e., at first mating in the previous study and at 24 hours after the first delivery in the current study, and that could affect to the values of immunological parameters.

Cytokines, C-reactive protein (CRP) and Cortisol

Cortisol concentration is used as a biochemical indicator of stress and pain (Stilwell et al., 2009). In the present study, the cortisol concentrations in the high and the low lines were higher than the normal range of cortisol concentration (2.6 – 3.8 μg/dL) for rabbits reported by (Washington and Van Hoosier, 2012). This may be due to the moment of recording.

Indeed, the samples were collected 24 hours after parturition. It is known that parturition is considered as one of the most stressful and painful events for the dam (Mainau and Manteca, 2011). Such a stressful event could increase the cortisol concentration up to several hours postpartum (Gladden et al., 2018). This finding implies that both lines were under similar level of stress.

It is well known that a high level of stress leads to dysregulation of the immune system (Webster et al. 2002), increasing predisposition to disease (Glaser and Kiecolt-Glaser, 2005). Inflammation is a biological response of the immune system that can be triggered by variety of factors, including pathogens and damaged cells (Liu et al., 2017). In order to evaluate the inflammatory process in both divergent lines, i.e. their susceptibility to diseases, we assessed the plasma levels of two cytokines as, interleukin-6 and tumor necrosis factor alpha, and C-reactive protein. Interleukin-6 (IL-6) is a pro-inflammatory cytokine, partly produced by the combined action of IL-1β and TNF-α, has effect on inflammation and immune response (Tanaka et al., 2014). TNF-α is a cytokine with pro-inflammatory activities produced by macrophages and have an important role in the innate defence mechanism (Amanda et al., 2009). A higher susceptibility to diseases is related with a higher concentration of TFN-α (Bruunsgaard et al., 1999). Both divergent lines were exposed to the same environment. However, the high line showed higher concentration of TNF-α than the low line. A high level of TFN-α agrees with higher inflammatory response in the high line, and therefore a higher susceptibility to diseases in this line.

C-reactive protein (CRP) is an acute-phase protein and an important etiological factor in inflammation (Jialal et al., 2004). Its production is mainly hepatic, by hepatocytes as a response on stimulation with IL-6, TNF-α and IL-1β (Reviewed by Stoner et al., 2013). Thus, CRP level in blood is considered as an inflammatory biomarker (Baumeister et al., 2016). Female rabbits of the high line showed greater CRP concentration. A higher basal CRP concentration in this line would confirm a higher sensitivity to disease, to the presence of chronic inflammation, and to a lesser tolerance to usual microorganisms in the farm microenvironment (Rauw, 2012; Markanday, 2015; Del Giudice and Gangestad, 2018). We note that selection for litter size environmental variability increased the difference in CPR between lines from 8^{th} generation (5.6 µg/ml, Argente et al., 2019) to 13^{th} generation (38.1 µg/ml), in agreement with a correlated response to selection for litter size environmental variability on animal's susceptibility to diseases.

Biochemical parameters

Infection and inflammation seem to have consequences on liver metabolism in order to reduce this inflammation (Sharma et al., 2018). We assessed biochemical indicators relationship between liver health and inflammation in both lines.Notice that delivery is a stressful event to does which could affect to biochemical parameters concentrations. However, the levels of bilirubin, cholesterol, ALP, GCT, BA, ALB and BUN in our lines were within the wide range of values reported in rabbits by Washington and Van Hoosier (2012) and Leineweber et al. (2018) (3.4 - 8.5 µmol/L, 0.3 - 3.0 µmol/L, 9.1 – 94.6 IU/L, 2.5 – 14.5 IU/L, 0.7 – 19.6 µmol/L, 25 - 50 g/L and 6.14 -8.38 µmol/L, respectively). When we compared both lines, the concentrations of bilirubin and cholesterol were higher in the high line. According to Fan et al. (2015), an increase in the concentration of cholesterol in rodents is a response to the production of inflammatory cytokines (mainly TNF-α and IL-1). Bilirubin is an endogenous antioxidant which promotes the lipid peroxidation prevention (Zhao et al., 2019). Inoguchi et al. (2016) reported that bilirubin plays a protective role against chronic inflammation. Taking together, it seems that high basal concentrations of cholesterol and bilirubin are related to a higher sensitivity to inflammation and susceptibility to diseases in the high line. Gamma glutamyl transferase (GGT) is an inflammation regulator which increases first in the case of a hepatic disorder (Elitok, 2012). Females from the high line showed higher GGT concentration than those from the low line; this would suggest a liver dysfunction and high susceptibility to diseases (Koenig and Seneff, 2015). The high line has a higher alkaline phosphatase (ALP) than the low line. ALP is a good indicator of liver diseases and general health (Kim et al., 2008). An increased concentration of ALP is an indication of liver dysfunction and a disruption in the inflammatory system (Dirksen et al., 2016). These results agree with the greater susceptibility to diseases of this line.
Recently a genome-wide association study has been performed on our lines, identifying several genes with functionality in the immune system and stress (Casto-Rebello et al., 2020). This finding corroborates the decisive role of the immune system in the environmental variation of litter size.

CONCLUSIONS

Our study shows the highline having higher inflammatory responseunder stressful situation as 24 hours after delivery, and consequently this line displays a greater susceptibility to diseases

and stress. Therefore, selection for litter size environmental variability can be a useful way to improve animal welfare.

FUNDING

This research was supported by Projects AGL2017-86083, C2-1-P and C2-2-P, funding by Ministerio de Ciencia e Innovación (MIC)-Agencia Estatal de Investigación (AEI) and el Fondo Europeo de Desarrollo Regional (FEDER) "Una manera de hacer Europa"

REFERENCES

Amanda M, Elena S, Mauricio A and Rajko R 2009. TNF-αin tuberculosis: A cytokine with a split personality. Inflamm Allergy Drug Targets 8, 53-62.

Argente MJ, García ML, Zbyňovká K, Petruška P, Capcarová M and Blasco A 2019. Correlated response to selection for litter size environmental variability in rabbit's resilience. Animal 13, 2348-2355.

Baumeister D, Akhtar R, Ciufolini S, Pariante CM and Mondelli V 2016. Childhood trauma and adulthood inflammation: a meta-analysis of peripheral C-reactive protein, interleukin-6 and tumour necrosis factor-α. Mol. Psychiatry 21, 642-649.

Blasco A 2017. Bayesian data analysis for animal scientists. New York: Springer.

Blasco A, Martínez-Álvaro M, García ML, Ibáñez-Escriche N and Argente MJ 2017. Selection for environmental variance of litter size in rabbit. Genet Sel Evol 49, 48-56.

Bodin L, Bolet G, García M, Garreau H, Larzul C and David I 2010b. Robustesse et canalisation: vision de généticiens. INRA Prod Anim 23, 11-22.

Broom DM 2008. Welfare assessment and relevant ethical decisions: key concepts. Ann. Rev. Biomed. Sci 20, 79-90.

Bruunsgaard H, Andersen-Ranberg K, Jeune B, Pedersen AN, Skinhøj P and Pedersen BK 1999. A high plasma concentration of TNF-α is associated with dementia in centenarians. Journal of Gerontology series A Biologial Sciences and Medical Sciences 54, 357-364.

Casto-Rebollo C, Argente MJ, García ML, Pena R and Ibáñez-Escriche N 2020. Identification of functional mutations associated with environmental variance of litter size in rabbits. Genet Sel Evol 52: 22.

Del Giudice M and Gangestad M 2018. Rethinking IL-6 and CRP: Why they are more than inflammatory biomarkers, and why it matters. Brain, Behavior, and Immunity 70, 61-75.

Dirksen K, Verzijl T and Van den Ingh TS 2016. Hepatocyte derived micro RNAs as sensitive serum biomarkers of hepatocellular injury in Labrador retrievers. V et J 211, 75-81.

Elitok B 2012. Reference values for heamatological and biochemical parameters in Saanen goats breeding in Afyonkarahisar province. Kocatepe Veterinary Journal 5, 7-11.

Fan J, Kitajima S, Watanabe T, Xu J, Zhang J, Liu E and Chen YE 2015. Rabbit models for the study of human atherosclerosis: from pathophysiological mechanisms to translational medicine. Pharma Col. Ther 146, 104-119.

Furze RC and Rankin SM 2008. Neutrophil mobilization and clearance in the bone marrow. Immunology 125, 281-288.

Gladden N, McKeegan D, Viora L and Ellis AK 2018. Postpartum ketoprofen treatment does not alter stress biomarkers in cows and calves experiencing assisted and unassisted parturition: a randomised controlled trial. Veterinary Record 10.1136/vr. 104913.

Glaser R and Kiecolt-Glaser JK 2005. Stress-induced immune dysfunction: implications for health. Nature Rev Immunol 5, 243-251.

Ihedioha J, Okorie I, Kanu CO and Iwuogu UM 2008. Leucocyte alterations associated with continual subacute blood loss. Proceedings of 33rd Annual Conference of Nigerian Society of Animal Production held in Ogun State, Nigeria.

Inoguchi T, Sonoda N and Meada Y 2016. Bilirubin as an important physiological modulator of oxidative stress and chronic inflammation in metabolic syndrome and diabetes: a new aspect on old molecule. Diabetol Int 7, 338-341.

Jialal I, Devaraj S and Venugopal SK 2004. C-reactive protein: risk marker or mediator in atherothrombosis? Hypertension 441, 6-11.

Kim W, Flamm SL, Di Bisceglie AM and Bodenheimer HC 2008. Serum activity of alanine amino transferase (ALT) as an indicator of health and disease. Hepatology 47, 1363-1370.

Koenig G and Seneff S 2015. Gamma-glutamyl transferase: a predictive biomarker of cellular antioxidant in adequacy and disease risk. Dis Markers 818570.

Leineweber C, Müller E and Marschang RE 2018. Blood reference intervals for rabbits (Oryctolagus cuniculus) from routine diagnostic samples. Bestimmung von Blutreferenzwerten für Kaninchen (Oryctolagus cuniculus) aus Routine diagnostic proben. Tierarztl Prax Ausg K Kleintiere Heimtiere 46, 393-398.

Lidfors L and EdströmT 2010. The Laboratory Rabbit. In The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals, 8th ed.; Hubrecht, R., Kirkwood, J., Eds. Wiley-Blackwell: West Sussex, UK 399-417.

Liu YA, Wang YX and Jiang CL 2017. Inflammation: The Common Pathway of Stress-Related. Diseases 11, 316-327.

Lockwood KM, Marsland AL, Cohen M and Gianaros PJ 2016. Sex Differences in the association between stressor-evoked interleukin-6 reactivity and C-reactive protein. Brain. Behav. Immun 58, 173-180.

Lung LHDS, Carvalheiro R, Neves HHDR and Mulder HA 2020. Genetics and genomics of uniformity and resilience in livestock and aquaculture species: A review. J Anim Breed Genet 137, 263-280.

Ma TX and Gao Y 2020. New insights into roles of basophils in initiating T helper type 2 immunity. Chinese Herbal Medicines 12, 14-18.

Mainau E and Manteca X 2011 Pain and discomfort caused by parturition in cows and sows. Appl. Anim. Behav. Sci 135, 241-251.

Mahgoub O, Kadim IT, Tageldin MH, Al-Marzooqi WS, Khalaf SQ and Ali AA 2008. Clinical profile of sheep fed non-conventional feeds containing phenols and condensed tannins. Small Ruminant Research 78, 115-122.

Markanday A 2015. Acute phase reactants in infections: evidence-based review and a guide for clinicians. Open Forum Infectious Diseases 2, 1-7.

Mbanasor UU, Anene BM, Chinezie AB, Nnaji TO, Eze JI and Ezekwe AG 2003. Haematology of normal trypanosome infected Muturu cattle in southeastern Nigeria. Nigerian Jour. Anim. Prod 30, 236-241.

Moore MD, Zimmerman K and Smith AS 2015.Hematologicalassessment in pet rabbits blood sample collection and blood cell identification. Vet. Clin. North. Am. Exot. Anim. Pract18, 9-19.

Mormede P, Boisseau-Sowinski L, Chiron J, Diederich C, Eddison J, Guichet JL, Le Neidre P and Meunier-Salaun MC 2018. Bien-être animal: context, definition, évaluation. INRA Prod. Anim 31, 145-162.

Otero RW, Velasco H and Sandoval H 2009. The protective role of bilirubin in human beings. Revista Colombiana de Gastroenterología 24, 293–301.

Rauw WM 2012. Immune response from a resource allocation perspective. Front. Genet 3, 267.

Scrivo R, Vasile M, Bartosiewicz I and Valesini G 2011. Inflammation as “common soil” of the multifactorial diseases. Auto immun Rev 10, 369-374.

Sharma A, Tate M, Mathew G, Vince JE, Ritchie RH and De Han JB 2018. Oxidative Stress and NLRP3-Inflammasome Activity as Significant Drivers of Diabetic Cardiovascular Complications: Therapeutic Implications. Front. Physiol 9: 114.

Stilwell G, Carvalho RC, Lima MS and Broom DM 2009. Effect of caustic paste disbudding, using local anaesthesia with and without analgesia, on behaviour and cortisol of calves. Appl. Anim. Behav. Sci 116, 35-44.

Stoner L, Lucero AA, Palmer RB, Jones ML, Young MJ and Faulkner J 2013. Inflammatory biomarkers for predicting cardiovascular disease. Clinical Biochemistry 46, 1353-1371.

Tanaka T, Narazaki M and Kishimoto T 2014. IL-6 in inflammation, immunity, and disease. Cold Spring Harb. Perspect. Biol 6:a016295.

Washington IM and Van Hoosier G 2012. Clinical Biochemistry and Hematology. In The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents. Suckow, M.A., Stevens K.A., Wilson R.P. Eds: Elsevier 57–116.

Webster JL, TonelliL and Sternberg EM 2002. Neuroendocrine regulation of immunity. Ann Rev Immunol 20, 125-163.

Zhao X, Li L, Li X, Li J, Wang D and Zhang H 2019. The relationship between serum bilirubin and inflammatory bowel disease. Hindawi: Mediators of Inflammation: 5256460.

Chapter 4. Preliminary metagenomics analyses in two lines of rabbit selected divergently for litter size environmental variability

ABSTRACT

A divergent selection experiment for the environmental variance of litter size variance was carried out in rabbits during thirteen generations. The aim of study was to analyse the microbial genes related to the environmental variability of litter size, in order to separate the high and the low line. At 22 weeks of age, samples were taken from the cecum content of 34 females of the high line and 36 females of the low line to extract bacterial DNA. Bacterial genome sequencing was performed by paired-end (2x150) in Illumina NextSeq 550. A total of 3046 microbial genes were identified. The filtering of variables according to their prevalence across samples was performed using the R package PIME. Subsequently, the zeros were imputed, and the weighted centered log-ratio (wCLR) normalisation was applied to treat the compositional data. Finally, the multivariate analysis using partial least square discriminant analysis (PLS-DA) was performed to assess the relationships microbial genes between the high and the low line. The PLS-DA score plot showed that the projection of data on the four latent variables had a good adjustment coefficient (R^2=0.85) and a good cross-validation (Q^2=0.52). The loading plot shows how the variables relate to each line and that the two lines are negatively correlated. In conclusion, multivariate analysis using PLS-DA allowed separating the two lines according to the microbial genes associated with the environmental variability of litter size. These findings provide support for the effect of host genotype on microbiome modification.

Keywords: Metagenome,environmental variability, rabbits, multivariate analysis, PLS-DA.

INTRODUCTION

The microbiota may play a crucial role in the stress level and health condition of the host (Sekirov et al., 2010). Metagenomics allows analysing all genomes from a community, being a useful tool for identifying the microbial diversity and its functions. In recent years, the rapid development of next-generation sequencing (NGS) techniques has allowed the reduction of the economic cost and sequencing time, helping to perform the studies of the gut microbiota in livestock animals (Thomas et al., 2012). NGS generates large volumes of sequence data containing genetic information (Metzker, 2010), and this requires the development of complex bioinformatics and statistical methods (Dhariwal et al., 2017). Metagenomic data is considered as compositional data for proceeding to its analysis. The compositional nature of

the data comes from the fact that they arenon-negative data that have been expressed relative to a fixed total (usually as proportions summing to 1 or percentages summing to 100%) (Greenacre, 2018).

The statistical analysis of metagenomic relative abundance data usually starts with the imputation of zeros (if it is necessary), followed by the normalisation of the data. The most appropriate way to analyse metagenomic data is to use the relative abundance of taxa or genes. This makes it easier to interpret the bioinformatics tools used. Taking into account the compositional data, several data transformation approaches have been proposed such as the centered log-ratio transformation (CLR) (Gloor and Reid, 2016). The last step is a multivariate analysis using the partial least square discriminant analysis (PLS-DA) that can visualize diversity measures through ordination plots (Calle, 2019). The PLS method tries to find the maximum correlation between the response variables and the predictors. PLS performs particularly well when the predictors are correlated and makes it possible to simplify the interpretation between the variables and the predictors, this is the reason why this method can be envisaged for metagenomic data.

A divergent selection experiment for the environmental variance of litter size at birth was performed in rabbits, after ten generations of selection the lines showed a remarkable divergent response (2.7 kits^2 in the low line vs. 4.4 kits^2 in the high one) (Blasco et al., 2017). Besides, the high line showed higher inflammatory response and susceptibility to infectious and diseases (Argente et al., 2019; Beloumi et al., 2020). This difference in susceptibility to diseases may be related to the microbiome.

The aim of this study was to analyse the existence of microbial genes associated withthe environmental variability of litter size that allows us to separate the high and the low line, using the partial least square discriminant analysis (PLS-DA).

MATERIAL AND METHODS

Ethics statement

All experimental procedures were approved by the Miguel Hernández University of Elche Research Ethics Committee, according to Council Directives 98/58/EC and 2010/63/EU (reference number 2017/VSC/PEA/00212).

Animals

A divergent selection experiment for environmental variance of litter size was performed in rabbits during 13 generations at the Universidad Miguel Hernández of Elche. Each divergent line had approximately 125 females and 25 males per generation. Selection was based on the phenotypic variance of litter size of each doe, after correcting litter size for both year-season and parity-lactation status (first parity, andlactating or not at mating in other parities). The details of the experiment are in Blasco et al. (2017). A total of seventy 22-week-old female rabbits from the 13th generation of selection were used for this experiment, 34 from the high line and 36 from the low line. Rabbits were fed with a standard commercial diet (17% crude protein, 16% fiber, 3.5% fat, Nutricun Elite Gra®, De HeusNutrición Animal, La Coruña, Spain). Food and water were provided *ad libitum*. They were under a constant photoperiod of 16:8 h and controlled ventilation. Does were housed in individual cages (37.5 cm x 33 cm x 90 cm). The experiment took place from March to July.

Sample collection

Animals were slaughtered, after 6 hours of feed deprivation.After slaughter and bleeding, caecum content samples were collected into tubes, homogenized, and divided into aliquots. The aliquots were immediately frozen using liquid nitrogen and then stored at -80ºC until posterior analysis.

DNA extraction and sequencing

The extraction of bacterial DNA from caecum samples was performed by mechanical homogenization with silica balls to release the genetic material, and with the DNeasy powerSoil kit N. 12888-100 to isolate the DNA. The massive sequencing of the samples was done by the company FISABIO (Valencia, Spain). DNA libraries were generated following the Nextera XT Illumina protocol using Nextera XT Library Prep kit (Illumina, Inc., San Diego, CA, USA). To initiate the protocol, 0.2 ng/µl of purified gDNA was taken from each sample. The mutiplexing step was performed using Nextera XT Index Kit. The libraries were sequenced using a 2x150pb paired-end run NextSeq high output reagent kit v2.5 on a NextSeq500 Sequencer according to manufacturer's instruction.

Bioinformatics analysis

The fastq files obtained after sequencing were filtered for quality check control in order to remove low- quality nucleotides prinseq-lite program (Schmieder and Edwards, 2011). Sequences were joined using FLASH program (Magoč and Salzberg, 2011). Reads from the host have been eliminated by mapping the reads with high quality against the reference rabbit genome database using the software Bowtie2 v2.3.4.3 (Langmead and Salzberg, 2012) with end-to-end read alignment.Sequences not contaminated by the host were analysed with the SqueezeMeta sequential method (Tamames and Puente-Sánchez, 2019). This pipeline covers all the steps for metagenomic analysis: quality filtering through the Trimmomatic program (Bolger et al., 2014), assembly of readings, gene prediction on contigs with Prodigal (Hyatt et al., 2010) and homology search against functional databases with Diamond software (Buchfink et al., 2015). The database used for the functional annotation was the available version of KEGG (Kanehisa and Goto, 2000).

Then, the functional annotation abundance matrice, which includes all individuals with the corresponding KEGG (microbial genes) and counts, was obtained. The relative abundances of the genes were estimated per animal.

STATISTICAL ANALYSIS

Preliminary analysis

The percentage of 0 counts has been calculated for all individuals in order to detect individuals having a high percentage of 0 counts. Then, the database was treated to remove low prevalence microbial genes in each line across all samples, keeping only genes shared at some level of prevalence using PIME (Prevalence Interval for Microbiome Evaluation) R package (Roesch et al., 2019). The R package PIME allows filtering microbial genes according to their prevalence across all samples. This package allowed us to filter the dataset per variable using different prevalence levels from 5% to 95% with increments of 5% for each level.

Data treatment

The compositional nature of metagenomic data requires a particular statistical approach. The process of analysing and the statistical procedures are illustrated in Figure 2.

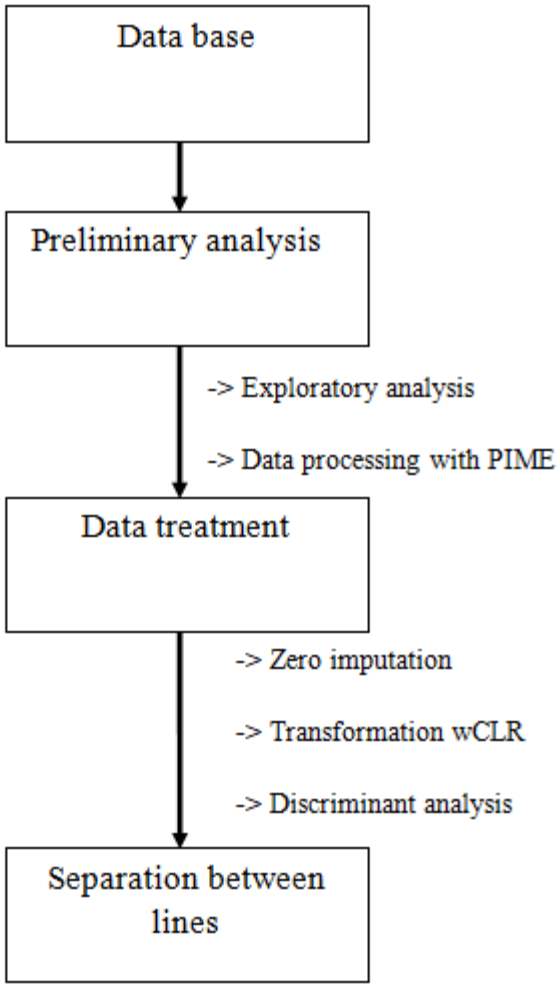

Figure 2. The process of analyzing metagenomic data.

- **Zero imputation**

Data with zero counts are presented in a large number of variables (genes). There are two types of zeros: essential zeros and missing data (Martin-Fernandez et al., 2000). Essential zeros are a real data indicating that there is not count in this variable; however, missing data are caused by the instrument that has not been sufficiently sensitive to detect counts. We cannot distinguish between missing data and essential zeros.

Compositional data are non-negative data, expressed as percentage and sum up to a constant (1 or 100%), and that can be transformed in real data using logarithms (Bacon-Shone, 2011). Furthermore, these types of data have an asymmetric distribution, so we use logarithms to make the distributions more symmetric. This transformation cannot be carried out with zero values. So, zero values must first be imputed if the data are going to be transformed using

logarithms. Imputing zeros is the process of replacing missing data or essential zeros with superseded values close to zero. To impute, a Bayesian-Multiplicative Replacement procedure was used (Martín-Fernández et al., 2015) using the cmultRepl function in R package zCompositions (Palarea-Albaladejo and Martín-Fernández, 2015).

- **Transformation wCLR**

As previously mentioned, metagenomic data are compositional data. Microbial genes expressed as relative abundances, were transformation using weighted centred log ratio (wCLR), expressed as:

$$wCLR\left(x_{ij}\right) = \log \frac{x_{ij}}{\prod_{1}^{n} x_{ij}^{w_j}} = \log\left(x_{ij}\right) - \sum_{1}^{n} w_j \log\left(x_{ij}\right) \quad (3)$$

$$w_j = \frac{\sum_{i=1}^{n} x_{ij}}{n} \quad (4)$$

where w_j is the weight, x_{ij} is the relative abundance of each variable, n is the number of samples, i is the sample and j is the total number of microbial genes.

This transformation was performed using the CLR function in R package easyCODA (Greenacre, 2018). The weighted CLR allow to downplay the variables that have little count (low relative abundance), and give importance to those that have more counts.

- **Discriminant analysis**

Partial least square discriminant analysis (PLS-DA) was performed on the wCLR of different prevalence interval data using SIMCA software (Umetrics, Umea, Sweden).We considered the lineas the categorical response and microbial genes as predictors. The objective of this analysis was to assess the relationships between microbial genes and lines. Determining the optimal number of latent components is an important step in building a PLS-DA model. This choice is commonly performed by using cross-validation. This method is based on repeat the regression several times on subsets of data and predict each time the values of the missing subsets. The outcomes from this procedure are the adjustment coefficient (R^2) and the cross-validation (Q^2). The equations for R^2 and Q^2 are:

$$R^2 = 1 - (RSS / TSS) \quad (5)$$

$$Q^2 = 1 - (PRESS / TSS) \quad (6)$$

where *RSS* is the sum of squared residuals, *TSS* is the total sum of squares, and *PRESS* is the predictive residual sum of the squares. R^2 and Q^2 are good parameters to indicate until which component to include in the model.

The criterion used to select variables (genes) was the VIP parameter (Variable Importance in Projection). VIP is a parameter that measures the importance of variables in a PLS model with many components(Galindo-Prieto et al., 2014). The VIP calculation is given in the following equation:

$$\text{VIP} = \sqrt{k \times \frac{\sum_{a=1}^{A} W_a^2 \times TSS\,(a)}{TSS\,(A)}} \tag{7}$$

where, W_a^2 is the squared PLS weight, TSS (a) is the sum of squares explained by component a, TSS (A) is the sum of squares explained by the model with A components, A is the total number of components, and k is the total number of variables. Only variables with VIP > 1 were considered important in the projection of the latent structure (Chong and Jun, 2005; Galindo-Prieto et al., 2014). If all variables have the same contribution on the model, all their VIPs will be equal to 1. Due to this reason, it is preferred to considervariables with VIP > 1 as the most relevant variables (Galindo-Prieto et al., 2014).

RESULTS AND DISCUSSION

Preliminary analysis

A total of 3046 microbial geneswere identified and kept for analysing. The distribution of genes with 0 counts for all individuals is shown in Figure 3; we can conclude that the individual 69 is an outlier since it has a high percentage of 0 counts (around 99%). Therefore, this animal has been eliminated because its presence can alter the results.

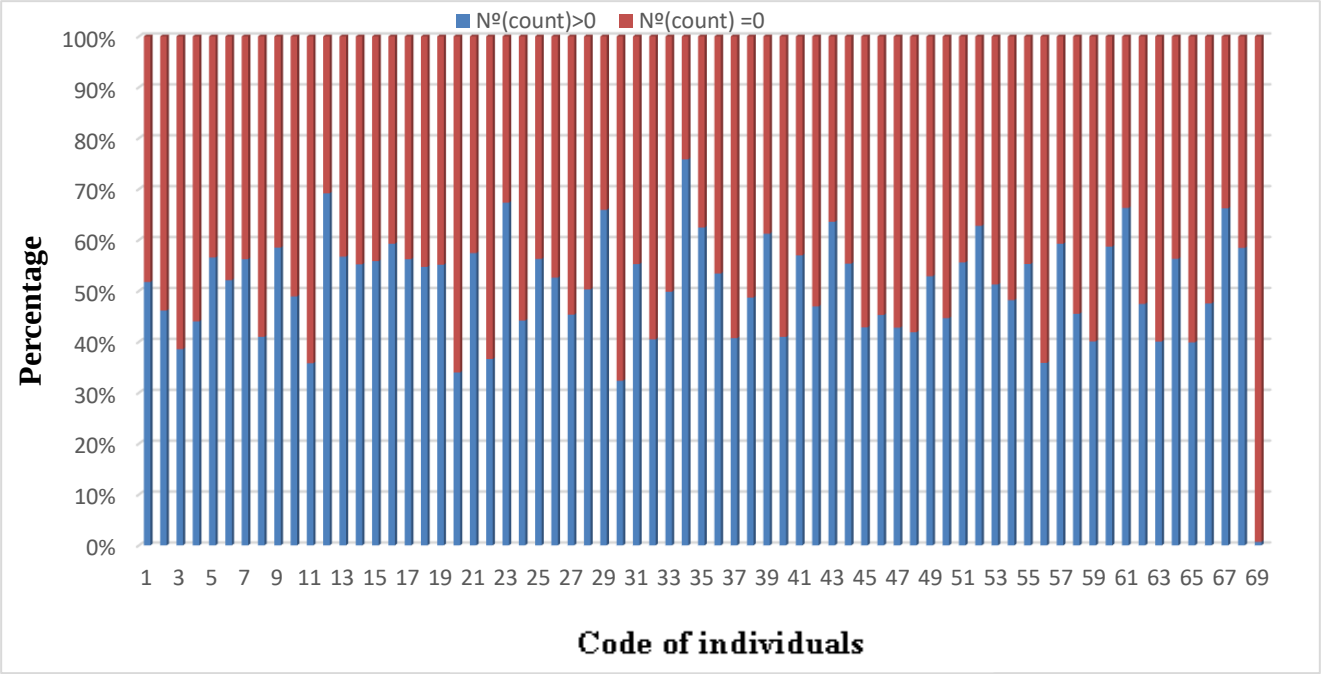

Figure 3. Distribution of microbial genes with 0 counts according to individuals.

Table 7 shows the three prevalences chosen according to the severity of the filtering. The prevalence 5% represents the light filtering, the 50% represents the intermediate filtering while the 95% interval represents the severe filtering. A prevalence of 5% indicates that at least one of two lines must have more than 5% of the data that they were not zero for each of the variables. It is noted that the number of variables decreases with increasing the prevalence.

Table 7. Number of variables, percentage of zeros (% Zeros) and the error of classification (OOB) obtained with the package PIME (Prevalence Interval for Microbiome Evaluation) of R for the prevalence 5%, 50% and 95%.

	Prevalence5%	Prevalence 50%	Prevalence 95%
Number of variables	2655	1526	976
% Zeros	41.37	10.83	1.05
OOB (%)	45.59	2.94	0

The OOB error allows evaluating the classification performance of the model; the values can vary between 0% and 100% (Table 7). Higher OOB error indicates the low accuracy of the model in predicting differences among variables. In our case, the zero OOB is only obtained

with a prevalence of 95%. Prevalence 50% has relatively low OOB value (2.94%) while the prevalence 5% has a high OOB value (45.59%).

We performed a PLS-DA discriminant analysis with this database. Several outliers were detected (Figure 4). These animals were eliminated because they were out to the region of discrimination, in addition to having a high percentage of 0 counts.

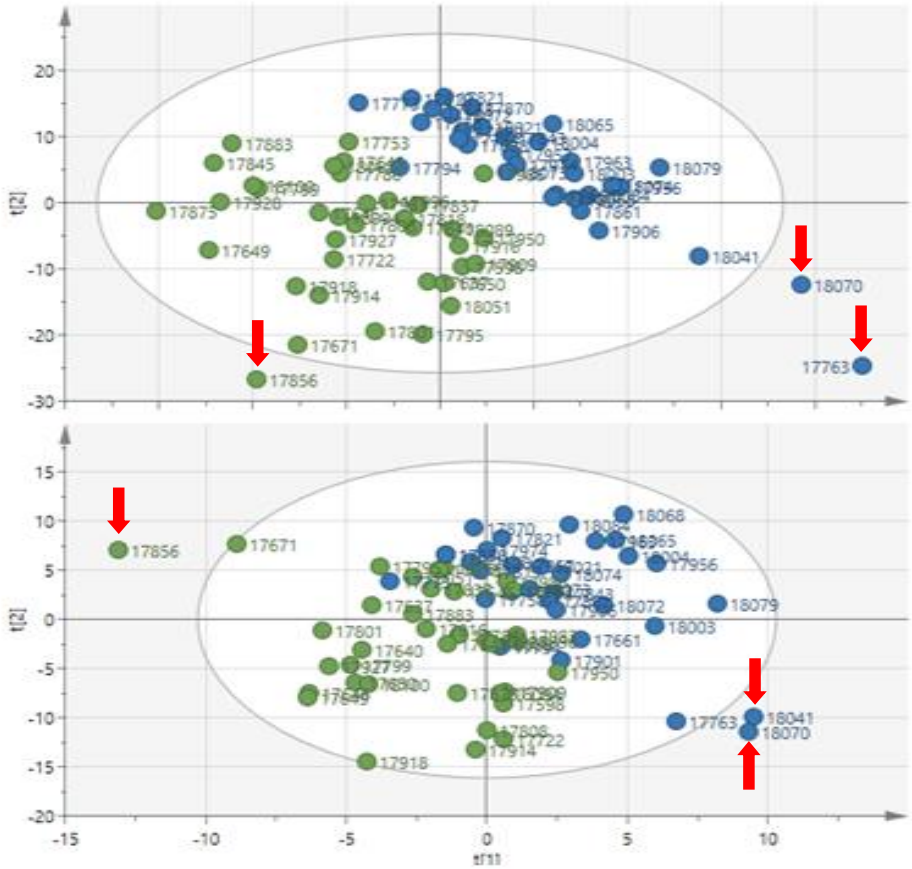

Figure 4. Score plot of prevalence 50% and 95% PLS-DA. Projection of the observations in the plane defined by the latent variables. Circles represent individuals. Colours: blue, high line; green, low line. The arrows in red are the outliers.

The filtering with the PIME package of the new database (without outliers) was done again. Table 8 shows the results for each prevalence applied in the dataset after removing outliers.

Table 8. Number of variables, percentage of zeros (% Zeros) and the error of classification (OOB) obtained with the package PIME (Prevalence Interval for Microbiome Evaluation) of R for the prevalence 5%, 50% and 95% after removing outliers.

	Prevalence5%	Prevalence 50%	Prevalence 95%
Number of variables	2620	1572	1034
% Zeros	39.69	10.50	0.71
OOB (%)	32.26	6.45	0

After removing outliers, the OOB obtained was 32.26% with prevalence 5%, 6.45% with prevalence 50% and 0 % with prevalence 95% (Table 8). The high OOB obtained with prevalence 5% is one of the reasons why we focused only on the other two prevalences. The second reason was to keep a number of variable high enough for analysing.

According to the Tables 7 and 8, after eliminating outliers, the number of variables increases forprevalence 50% and 95%. Indeed, the elimination of individuals with a high percentage of 0 counts makes possible to reduce the pressure and the percentage of 0 counts in certain variables, hence its retention in the model.

Discriminant analysis

We performed a partial least square discriminant analysis (PLS-DA) to associate microbial genes and lines. We did a PLS-DA analysis with two different prevalences (50% and 95%). The results of PLS-DA are presented in the Table 9.

Table 9. Number of variables, number of variables whose VIP is greater than 0.95 (Variable VIP > 0.95, adjustment coefficient (R^2) and cross-validation (Q^2) associated to PLS-DA with variables VIP > 0.95 after removing outliers.

	Prevalence 50%	Prevalence 95%
Number of variables	1572	1034
Variable VIP > 0.95	448	292
R^2	0.85	0.85
Q^2	0.52	0.47

The two prevalences 50% and 95% have the same value of R^2, but the prevalence 50% has slightly a better cross-validation (Q^2= 0.52), i.e., better accuracy in prediction of model, and in addition to having a greater number of variables than prevalence 95%. Hence, the prevalence 50% has been selected to follow the analyses and to select variables.

Table 10 shows the principal statistical parameters of PLS-DA for prevalence 50%. The choice of the optimal number of latent variables is made generally based on adjustment coefficient (R^2) and cross-validation (Q^2) values. In this case, from the second component, the values of R^2 are quite high, therefore the choice of the optimal number of latent variables is made according to the values of Q^2. The predictive ability for the first two components measured by the cumulative was insufficient ($Q^2 = 0.34$) and for the first four components the cumulative ($Q^2 = 0.52$). Hence, we chose to work with the first four components.

Table 10. Statistical parameters of PLS-DA for prevalence 50%.

Number of components	R^2	Q^2
1	0.4	0.04
2	0.67	0.34
3	0.78	0.39
4	0.85	0.52
5	0.92	0.64
6	0.97	0.74
7	0.98	0.78

R^2: the adjustment coefficient; Q^2: the cross-validation coefficient

The PLS-DA score plot showed that the projection of data on the four latent variables has a good adjustment coefficient ($R^2 = 0.85$). This projection explained 52% of the predicted variance from cross-validation ($Q^2 = 0.52$).

A good separation was observed between lines and confirmed by the score plot (Figure 5). The Hotelling T^2 ellipse shows two animals from the high line outside the 95% confidence region of the model. We did not eliminate these two individuals because they are relatively close to the region of discrimination, in addition to having a low percentage of 0 counts compared to the animals previously eliminated.

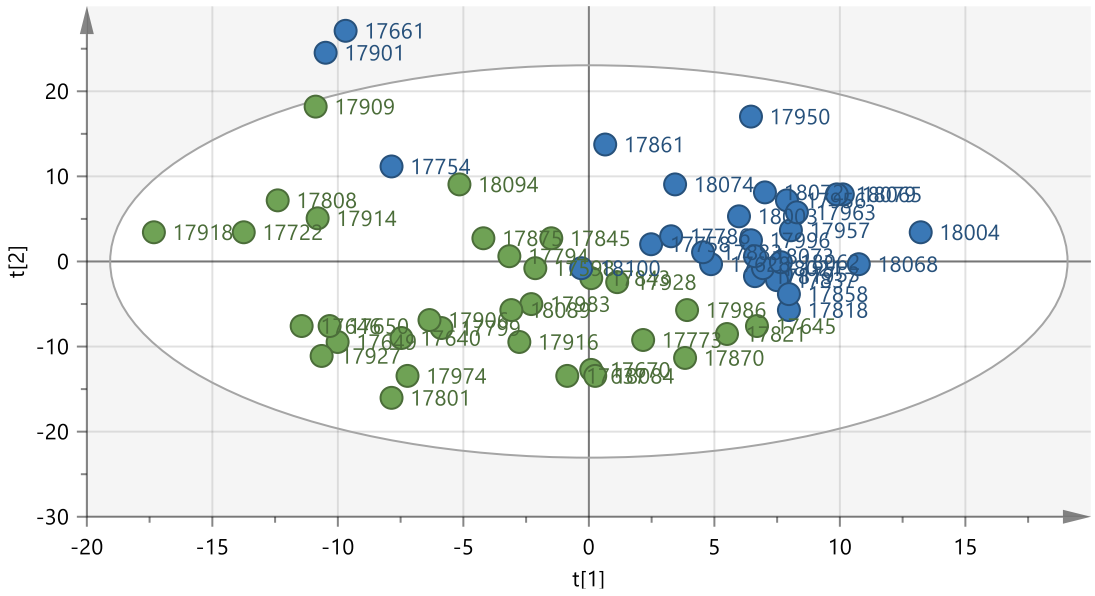

Figure 5. Score plot of prevalence 50% PLS-DA. Projection of the observations in the plane defined by the latent variables. circles represent individuals. Colours: blue, high line; green, low line.

The loading plot (Figure 6) shows how the variables relate to each line and that the two lines are negatively correlated.

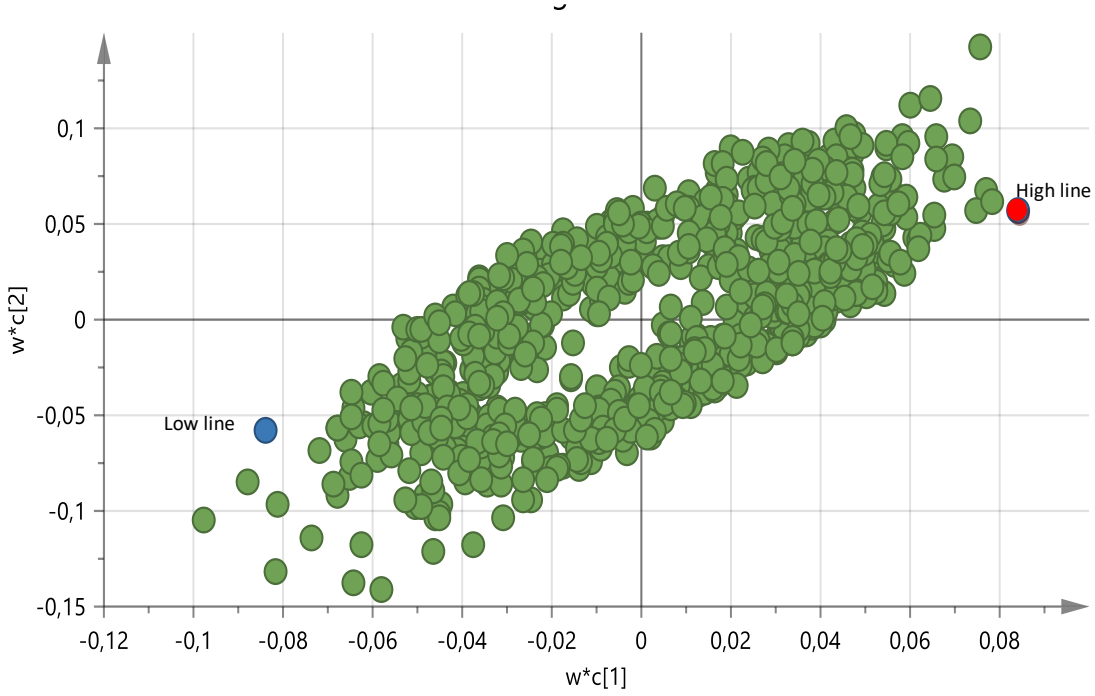

Figure 6. Loading plot of filtered 50% PLS-DA. Colours: green, microbial genes; red, high line; blue, low line.

VIP (variable importance in the projection) is a parameter to find which variable is most relevant for explaining the lines. VIP is equal to 1 means that all variables have the same contribution on the model. Due to this reason,it is preferred to use variables with VIP > 1 as the most relevant variables (Galindo-Prieto et al., 2014). But in our case, VIPs> 0.95 have been selected so as not to eliminate a large number of VIPs between 0.95 and 1 that can make the difference in the discrimination.

This work is part of a project that aims to identify microbial genes associated with environmental variability of litter size in rabbits. Environmental variance of litter size has a low heritability, then the associations between the trait and microbial genes may be mainly random. In order to resolve this problem, we use two selected lines divergently for the environmental variability of litter size (Blasco et al., 2017), and thus we can associate the genes to the trait directly. As both lines are under the same environmental, we can consider that the associations obtained are due to only genetic effects. But, the divergent selection can have also sampling effects caused in this case by genetic drift. Moreover, if an allele has an extreme frequency (tend towards 1 or tend towards 0) at the beginning of the selection, in the following generations, this frequency will change slightly in value. For example, if an allele for homogeneity has a frequency of 0.99, it cannot be separated in the two lines in subsequent generations.

The aim of our study was to analyse the microbial genes related to the environmental variability of litter size, in order to separate the high line and the low line using PLS-DA.Partial least square discriminant analysis (PLS-DA) was used to associate microbial genes and lines. PLS-DA is a useful statistical approach for discrimination when we have a larger number of predictors (Varmuza and Filzmoser, 2009). This is why recently, the PLS-DA start of being used in metagenomic and metabolomic analyses (Zang and Powers, 2012). In our case, the PLS-DA was able to discriminate between the two lines (high line and low line), and the loading plot (Figure 5) showed that the two lines were clearly separated. This discrimination capacity of the PLS-DA was confirmed by Clos-Garcia et al. (2020) comparing the metabolomics analysis of different groups of patients (healthy controls and colorectal cancer patients) in order toidentify early biomarkersfor disease progression.Furthermore, Gao et al. (2017) with a metagenomic analysis, also confirmed the ability of PLS-DA to separate the microbiome composition and genes between two groups of mice to determine the impact of diazinon exposure on the gut microbiome composition and its metabolic functions.

After this discrimination analysis, the most important KEGGs (according to the value of VIP) should be identified in order to know their biological and metabolic functions.

CONCLUSIONS

The PLS-DA approach usedto discriminate between the two divergent lines, allowed us to identify microbial genes that discriminate between linesassociated with environmental variability of litter size. These findings provide support for the effect of host genotype on microbiome modification. Further studies are needed toidentify the functions of these genes as well as the taxa responsible for the separation between lines.

FUNDING

This research was supported by Projects AGL2017-86083, C2-1-P and C2-2-P, funding by Ministerio de Ciencia e Innovación (MIC)-Agencia Estatal de Investigación (AEI) and el Fondo Europeo de Desarrollo Regional (FEDER) "Una manera de hacer Europa"

REFERENCES

Argente MJ, García ML, Zbyňovká K, Petruška P, Capcarová M and Blasco A 2019. Correlated response to selection for litter size environmental variability in rabbit's resilience. Animal 13, 2348-2355.

Bacon-Shone J 2011. "A Short History of Compositional Data Analysis," in Compositional Data Analysis: Theory and Applications ed. Pawlowsky-Glahn V and Buccianti A [NY: John Wiley and Sons], 3-11.

Beloumi D, Blasco A, Muelas R, Santacreu MA, García ML and Argente MJ 2020. Inflammatory Correlated Response in Two Lines of Rabbit Selected Divergently for Litter Size Environmental Variability. Animals 10: 1540.

Blasco A, Martínez-Álvaro M, García ML, Ibáñez-Escriche N and Argente MJ 2017. Selection for environmental variance of litter size in rabbits. Genet Sel Evol 49, 48-55.

Bolger AM, Lohse M and Usadel B 2014. Trimmomatic: a flexible trimmer for Illumina sequence data 30, 2114-2120.

Buchfink B, Xie C and Huson DH 2015. Fast and sensitive protein alignment using DIAMOND. Nat. Methods 12, 59-60.

Calle ML 2019. Statistical Analysis of Metagenomics Data. Genomics Inform 17, e6.

Chong IG and Jun CH 2005. Performance of some variable selection methods when multicollinearity is present. Chemometrics and Intelligent Laboratory Systems 78, 103-112.

Clos-Garcia M, Garcia K, Alonso C, Iruarrizaga-Lejarreta M, D'Amato M, Crespo A, Iglesias A, Cubiella J, Bujanda L and Falcón-Pérez JM 2020. Integrative Analysis of Fecal Metagenomics and Metabolomics in Colorectal Cancer. Cancers 12, 1142-1164.

Dhariwal A, Chong J, Habib S, King IL, Agellon LB and Xia J 2017. Microbiome Analyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Research 45, 180-188.

Galindo-Prieto B, Eriksson L and Trygg J 2014. Variable influence on projection (VIP) for orthogonal projections to latent structures (OPLS). Journal of Chemometrics 28, 623-632.

Gao B, Bian X, Mahbub R and Lu K 2017. Sex-specific effects of organophosphate diazinon on the gut microbiome and its metabolic functions. Environ Health Perspect 125, 198-206.

Gloor GB and Reid G 2016. Compositional analysis: a valid approach to analyze microbiome high-throughput sequencing data. Can J Microbiol 62, 692-703.

Gloor GB, Macklaim JM, Pawlowsky-Glahn V and Egozcue JJ 2017. Microbiome datasets are compositional: And this is not optional. Front. Microbiol 8, 1-6.

Greenacre M 2018. Compositional Data Analysis in Practice, Chapman & Hall / CRC Press.

Hyatt D, Chen G, Locascio PF, Land ML, Larimer FW and Hauser LJ 2010. Prodigal : prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 11:119.

Kanehisa M and Goto S 2000. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res 27, 29-34.

Langmead B and Salzberg SL 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357-359.

Magoč T and Salzberg SL 2011. *FLASH:* fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957-2963.

Martín-Fernández JA, Barceló-Vidal C and Pawlowsky-Glahn V 2000. Zero Remplacement in Compositional Data Sets. In: Kiers HAL, Rasson JP, Groenen PJF, Schader M (eds) Data Analysis, Classification, and Related Methods. Studies in Classification, Data Analysis, and Knowledge Organization. Springer, Berlin, Heidelberg.

Martín-Fernández JA, Hron K, Templ M, Filzmoser P and Palarea-Albaladejo J 2015. Bayesian-multiplicative treatment of count zerosin compositional data sets. Stat. Modelling 15, 134-158.

Metzker ML 2010. Sequencing technologies — the next generation. Nature Reviews Genetics 11, 31-46.

Palarea-Albaladejo J and Martín-Fernández JA 2015. zCompositions – R package for multivariate imputation of left-censored data under a compositional approach. Chemom. Intell. Lab. Syst 143, 85-96.

Roesch LFW, Dobbler PT, Pylro VS, Kolaczkowski B, Drew JC and Triplett EW 2019. PIME: A package for discovery of novel differences among microbial communities. Mol Ecol Resour 20, 415-428.

Schmieder R and Edwards R 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27, 863-864.

Sekirov I, Russell SL, Antunes LC and Finlay BB 2010. Gut microbiota in health and disease. Physiol Rev 90, 859-904.

Tamames J and Puente-sánchez F 2019. SqueezeMeta , A Highly Portable , Fully Automatic Metagenomic Analysis Pipeline 9, 1–10.

Thomas T, Gilbert J and Meyer F 2012. Metagenomics a guide from sampling to data analysis. Microb Informatics Exp 2: 3.

Varmuza K and Filzmoser P 2009. Introduction to Multivariate Statistical Analysis in Chemometrics. CRC Press, Taylor & Francis Group.

Zang B and Powers R 2012. Analysis of bacterial biofilms using NMR-based metabolomics. Future Med Chem 4, 1273-1306.

Chapter 5. General discussion

A divergent selection experiment for environmental variance of litter size at birth was carried out successfully in rabbits during thirteen generations (Blasco et al., 2017). Environmental variance of litter size has been related to response to stress and disease in females, which are related to welfare (Argente et al., 2019; Bergholf et al., 2019; Formoso-Rafferty et al., 2016). Inflammation is the immune system's response that is triggered to microbial invasion or tissue damage (Liu et al., 2017). Then, inflammatory response can be used to measure the susceptibility to diseases in the animal. Susceptibility to diseases and stress is closely related to the gut microbiota of the animal. Indeed, it is well known that gut microbiota has an important role in the development of the immune system and the activation of the hypothalamic-pituitary-adrenal (HPA) axis in the host. This study aimed to analyse the effect of selection for environmental variance of litter size on susceptibility to diseases, and to examine the existence of microbial genes associated withthe environmental variability of litter size that separates the high and the low line.

In the chapter 3, we found that the high lineis characterized by a major chronic inflammation and a large weakness of their immune system. Therefore, animals from the high line are more susceptible to diseases and infections than those from the low line, and consequently presenting a higher degree of variability in litter size. We can conclude thatselection for environmental variabilitymay have implications on animal welfare.

In the chapter 4, we performed an exploratory metagenomic study using the former lines. Metagenomic data analysis is complex and requires several steps (Thomas et al., 2012). After imputing the zeros, find the optimal prevalence interval and normalize the data, a PLS-DA analysis was performed to allow us to identify microbial genes that discriminate between lines. The two lines are separated according to the microbial genes, suggesting that selection for environmental variability of litter size intervenes in the modification of the microbiome.

After this preliminary analysis, additional studies will be necessary carried out to identify the most important KEGGs according to the VIP value, in order to know their biological and metabolic functions on susceptibility to diseases in our divergent lines for environmental variance of litter size.

Literature cited

Argente MJ, García ML, Zbyňovká K, Petruška P, Capcarová M and Blasco A 2019. Correlated response to selection for litter size environmental variability in rabbit'sresilience. Animal 13, 2348-2355.

Berghof TVL, Poppe M and Mulder HA 2019. Opportunities to improve resilience in animal breeding programs. Front. Genet 9: 692.

Blasco A, Martínez-Álvaro M, García ML, Ibáñez-Escriche N and Argente MJ 2017. Selection for environmental variance of litter size in rabbit. Genet. Sel. Evol 49, 48-56.

Formoso-Rafferty N, Cervantes I, Ibañez-Escriche N and Gutiérrez JP 2016. Correlated genetic trends for production and welfare traits in a mouse population divergently selected for birth weight environmental variability. Animal 10, 1770-1777.

Liu YZ, Wang YX and Jiang CL 2017. Inflammation: The Common Pathway of Stress-Related Diseases. Front Hum Neurosci 11, 316-327.

Thomas T, Gilbert J and Meyer F 2012. Metagenomics a guide from sampling to data analysis. Microb Informatics Exp 2: 3.

Varmuza K and Filzmoser P 2009. Introduction to Multivariate Statistical Analysis in Chemometrics. CRC Press, Taylor & Francis Group.

Zang B and Powers R 2012. Analysis of bacterial biofilms using NMR-based metabolomics. Future Med Chem 4, 1273-1306.

References

Abecia L, Martín-García AI, Martínez G, Newbold CJ and Yáñez-Ruiz DR 2013. Nutritional Intervention in Early Life to Manipulate Rumen Microbial Colonization and Methane Output by Kid Goats postweaning1. Journal of Animal Science 91, 4832–40.

Agea I, García ML, Blasco A and Argente MJ 2019. Litter Survival Differences between Divergently Selected Lines for Environmental Sensitivity in Rabbits. Animals 9: 603.

Amanda M, Elena S, Mauricio A and Rajko R 2009. TNF-αin tuberculosis: A cytokine with a split personality. Inflamm Allergy Drug Targets 8, 53-62.

Aoki-Kinoshita KF and Kanehisa M 2007. Gene Annotation and Pathway Mapping in KEGG. In: Bergman N.H. (eds) Comparative Genomics. Methods In Molecular Biology™, 396. Humana Press.

Arpaia N, Campbell C, Fan X, Dikiy S, Van JDV, Deroos P, Liu H, Cross JR, Pfeffer K and Coffer PJ 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 504: 451

Argente MJ, Calle EW, García ML and Blasco A 2017. Correlated response in litter size components in rabbits selected for litter size variability. Journal of Animal Breeding and Genetics 134, 505-511.

Argente MJ, García ML, Zbyňovká K, Petruška P, Capcarová M and Blasco A 2019. Correlated response to selection for litter size environmental variability in rabbit's resilience. Animal 13, 2348-2355.

Awasthi MK, Ravindran B, Sarsaiya S, Chen H, Wainaina S, Singh E, Tao L, Kumar S, Pandey A, Singh L and Zhang Z 2020. Metagenomics for taxonomy profiling: tools and approaches. Bioengineered 11, 356-374.

Bacon-Shone J 2011. "A Short History of Compositional Data Analysis," in Compositional Data Analysis: Theory and Applications ed. Pawlowsky-Glahn V and Buccianti A [NY: John Wiley and Sons], 3-11.

Baumeister D, Akhtar R, Ciufolini S, Pariante CM and Mondelli V 2016. Childhood trauma and adulthood inflammation: a meta-analysis of peripheral C-reactive protein, interleukin-6 and tumour necrosis factor-α. Mol. Psychiatry 21, 642-649.

Beloumi D, Blasco A, Muelas R, Santacreu MA, García ML and Argente MJ 2020. Inflammatory Correlated Response in Two Lines of Rabbit Selected Divergently for Litter Size Environmental Variability. Animals 10: 1540.

Berardini TZ, Mundodi S, Reiser L, Huala E, Garcia-Hernandez M,…and Rhue SY 2004. Funcional Annotation of the Arabidopsis Genome Using Controlled Vocabularies. Plant Physiol 135, 745-755.

Berghof TVL, Poppe M and Mulder HA 2019. Opportunities to improve resilience in animal breeding programs. Front. Genet. 9: 692.

Bienenstock J, Kunze W and Forsythe P 2015. Microbiota and the gut-brain axis. Nutri. Rev 73, 28-31.

Blasco A 2017. Bayesian data analysis for animal scientists. New York: Springer.

Blasco A, Martínez-Álvaro M, García ML, Ibáñez-Escriche N and Argente MJ 2017. Selection for environmental variance of litter size in rabbits. Genet Sel Evol 49, 48-55.

Blekhman R, Goodrich JK, Huang K, Sun Q, Bukowski R, Bell JT, Spector TD, Keinan A, Ley RE, Gevers D and Clark AG 2015. Host genetic variation impacts microbiome composition across human body sites. Genome Biology 16: 191.

Bodin L, Garcia M, Saleil G, Bolet G and Garreau H 2010a. Results of 10 generations of canalising selection for rabbit birth weight. Results of canalizing selection for rabbit birth weight. Proc. 9thWCGALP, Liepzig, Germany, 2-6 august 2010.

Bodin L, Bolet G, Garcia M, Garreau H, Larzul C and David I 2010b. Robustesse et canalisation: vision de généticiens. INRA Prod. Anim 23, 11-22.

Bolger AM, Lohse M and Usadel B 2014. Trimmomatic: a flexible trimmer for Illumina sequence data 30, 2114-2120.

Bonder MJ, Kurilshikov A, Tigchelaar EF, Mujagic Z, Imhann F, ... and Zhernakova DV 2016. The effect of host genetics on the gut microbiome. Nature genetics 48, 1407-1412.

Bouskra D, Brezillon C, Berard M, Werts C, Varona R, Boneca IG and Eberl G 2008. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature 456, 507-510.

Broom DM 2008. Welfare assessment and relevant ethical decisions: key concepts. Ann. Rev. Biomed. Sci 20, 79-90.

Bruunsgaard H, Andersen-Ranberg K, Jeune B, Pedersen AN, Skinhøj P and Pedersen BK 1999. A high plasma concentration of TNF-α is associated with dementia in centenarians. Journal of Gerontology series A Biologial Sciences and Medical Sciences 54, 357-364.

Buchfink B, Xie C and Huson DH 2015. Fast and sensitive protein alignment using DIAMOND. Nat. Methods 12, 59-60.

Calle EW, García ML, Blasco A and Argente MJ 2017. Correlated response in early embryonic development in rabbits selected for litter size variability. World Rabbit Science 25, 323-327.

Calle ML 2019. Statistical Analysis of Metagenomics Data. Genomics Inform 17: e6.

Carabaño R, Badiola I, Licois D and Gidenne T 2006. The digestive ecosystem and its control through nutritional or feeding strategies. Recent advances in rabbit sciences, 211-227.

Carabaño R, Piquer J, Menoyo D and Badiola I 2010. The digestive system of the rabbit. CAB International 2010. Nutrition of the rabbit, 2nd Edition.

Casto-Rebollo C, Argente MJ, García ML, Pena R and Ibáñez-Escriche N 2020. Identification of functional mutations associated with environmental variance of litter size in rabbits. Genet Sel Evol 52: 22.

Chong IG and Jun CH 2005. Performance of some variable selection methods when multicollinearity is present. Chemometrics and Intelligent Laboratory Systems 78, 103-112.

Clos-Garcia M, Garcia K, Alonso C, Iruarrizaga-Lejarreta M, D'Amato M, Crespo A, Iglesias A, Cubiella J, Bujanda L and Falcón-Pérez JM 2020. Integrative Analysis of Fecal Metagenomics and Metabolomics in Colorectal Cancer. Cancers 12, 1142-1164.

Colditz I and Hine B 2016. Resilience in farm animals: Biology, management, breeding and implications for animal welfare. Animal Production Science 56, 1961-1983.

Collins SM, Surette M and Bercik P 2012. The interplay between the intestinal microbiota and the brain. Nature reviews Microbiology 10, 735-42.

Combes S, Fortun-Lamothe L, Cauquil L and Gidenne T 2011. Piloter l'écosystème digestif du lapin : pourquoi, quand et comment. 14ème Journées de la Recherche Cunicole, 22-23 novembre 2011, Le Mans, France.

Combes S, Gidenne T, Cauquil L, Bouchez O and Fortun-Lamothe L 2014. Coprophagous behavior of rabbit pups affects implantation of cecal microbiota and health status. J. Anim. Sci 92, 652-665.

Crowley EJ, King JM, Wilkinson T, Worgan HJ, Huson KM, Rose MT and McEwan NR 2017. Comparison of the microbial population in rabbits and guinea pigs by next generation sequencing. PLoS One 12: e0165779.

De Vadder F, Kovatcheva-Datchary P, Goncalves D, Vinera J, Zitoun C, Duchampt D, Backhed F and Mithieux G 2014. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. Cell156, 84-96.

Del Giudice M and Gangestad M 2018. Rethinking IL-6 and CRP: Why they are more than inflammatory biomarkers, and why it matters. Brain, Behavior, and Immunity 70, 61-75.

Dhariwal A, Chong J, Habib S, King IL, Agellon LB and Xia J 2017. Microbiome Analyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Research 45, 180-188.

Dirksen K, Verzijl T and Van den Ingh TS 2016. Hepatocyte derived micro RNAs as sensitive serum biomarkers of hepatocellular injury in Labrador retrievers. V et J 211, 75-81.

Dodd D, Spitzer MH, Van Treuren W, Merrill BD, Hryckowian AJ, Higginbottom SK, Le A, Cowan TM, Nolan GP, Fischbach MA and Sonnenburg JL 2017. A gut bacterial pathway metabolizes aromatic amino acids into nine circulating metabolites. Nature 551, 648-652.

Elitok B 2012. Reference values for heamatological and biochemical parameters in Saanen goats breeding in Afyonkarahisar province. Kocatepe Veterinary Journal 5, 7-11.

Falconer D S and Mackay TCF 1996. Introduction to Quantitative Genetics (London, Longman).

Fan J, Kitajima S, Watanabe T, Xu J, Zhang J, Liu E and Chen YE 2015. Rabbit models for the study of human atherosclerosis: from pathophysiological mechanisms to translational medicine. Pharma Col. Ther 146, 104-119.

Fina M, Ibañez-Escriche N, Piedrafita J and Casellas J 2013. Canalization analysis of birth weight in Bruna delsPirineus beef cattle. J Anim Sci 91, 3070-3078.

Formoso-Rafferty N, Cervantes I, Ibañez-Escriche N and Gutiérrez JP 2015. Genetic control of the environmental variance for birth weight in seven generations of a divergent selection experiment in mice. Journal of Animal Breeding and Genetics, doi: 10.1111/jbg.12174.

Formoso-Rafferty N, Cervantes I, Ibañez-Escriche N and Gutiérrez JP 2016. Correlated genetic trends for production and welfare traits in a mouse population divergently selected for birth weight environmental variability. Animal 10, 1770-1777.

Formoso-Rafferty N, Chavez KN, Ojeda C, Cervantes I and Gutiérrez JP 2020. Selection Response in a Divergent Selection Experiment for Birth Weight Variability in Mice Compared with a Control Line. Animals 10: 920.

Furze RC and Rankin SM 2008. Neutrophil mobilization and clearance in the bone marrow. Immunology 125, 281-288.

Galindo-Prieto B, Eriksson L and Trygg J 2014. Variable influence on projection (VIP) for orthogonal projections to latent structures (OPLS). Journal of Chemometrics 28, 623-632.

Gao B, Bian X, Mahbub R and Lu K 2017. Sex-specific effects of organophosphate diazinon on the gut microbiome and its metabolic functions. Environ Health Perspect 125, 198-206.

Garreau H, Bolet G, Larzul C, Rovert-Granié C, Saleil G, SanCristobal M and Bodin L 2008. Results of four generations of a canalising selection for rabbit birth weight. Livest. Sci 119, 55-62.

Gidenne T, Combes S, Licois D, Carabaño R, Badiola I and Garcia J 2008. Ecosystème caecal et nutrition du lapin : interactions avec la santé digestive. INRA Prod. Anim 21, 239-250.

Gladden N, McKeegan D, Viora L and Ellis AK 2018. Postpartum ketoprofen treatment does not alter stress biomarkers in cows and calves experiencing assisted and unassisted parturition: a randomised controlled trial. Veterinary Record 10.1136/vr. 104913.

Glaser R and Kiecolt-Glaser JK 2005. Stress-induced immune dysfunction: implications for health. Nature Rev Immunol 5, 243-251.

Gloor GB and Reid G 2016. Compositional analysis: a valid approach to analyze microbiome high-throughput sequencing data. Can J Microbiol 62, 692-703.

Gloor GB, Macklaim JM, Pawlowsky-Glahn V and Egozcue JJ 2017. Microbiome datasets are compositional: And this is not optional. Front. Microbiol 8, 1-6.

Greenacre M 2018. Compositional Data Analysis in Practice, Chapman & Hall / CRC Press.

Gutiérrez JP, Nieto B, Piqueras P, Ibáñez N, Salgado C 2006. Genetic parameters for canalisation analysis of litter size and litter weight traits at birth in mice. Genetics Selection Evolution 38, 445-462.

Guyomar C 2018. Développement et applications d'outils d'analyse métagénomique des communautés microbiennes associées aux insectes. Doctoral thesis. Sciences agricoles. Université Rennes 1. NNT: 2018REN1B049f.

Hyatt D, Chen G, Locascio PF, Land ML, Larimer FW and Hauser LJ 2010. Prodigal : prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 11:119.

Hill WG and Mulder HA 2010. Genetic analysis of environmental variation. Genet.Res.Camb. 92, 381-395.

Ibáñez-Escriche N, Varona L, Sorensen D and Noguera JL 2008a. A study of heterogeneity of environmental variance for slaughter weight in pigs. Animal 2, 19-26.

Ibáñez-Escriche N, Moreno A, Nieto B, Piqueras P, Salgado C, Gutiérrez JP 2008b. Genetic parameters related to environmental variability of weight traits in a selection experiment for weight gain in mice; signs of correlated canalised response. Genetics Selection Evolution 40, 279-293.

Ihedioha J, Okorie I, Kanu CO and Iwuogu UM 2008. Leucocyte alterations associated with continual subacute blood loss. Proceedings of 33rd Annual Conference of Nigerian Society of Animal Production held in Ogun State, Nigeria.

Inoguchi T, Sonoda N and Meada Y 2016. Bilirubin as an important physiological modulator of oxidative stress and chronic inflammation in metabolic syndrome and diabetes: a new aspect on old molecule. Diabetol Int 7, 338-341.

Jialal I, Devaraj S and Venugopal SK 2004. C-reactive protein: risk marker or mediator in atherothrombosis? Hypertension 441, 6-11.

Kanehisa M and Goto S 2000. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res 27, 29-34.

Kamada N, Chen GY, Inohara N and Nuñez G 2013. Control of pathogens and pathobionts by the gut microbiota. Nat. Immunol 14, 685-690.

Kanehisa M, Goto S, Kawashima S, Okuno Y and Hattori M 2004. The kegg resource for deciphering the genome. Nucleic acids research 32, 277-280.

Kim M, Qie Y, Park J and Chang HK 2016. Gut microbial metabolites fuel host antibody responses. Cell Host Microbe 20, 202-214.

Kim TK, Thomas SM, Ho M, Sharma S, ... and Wilson BA 2009. "Heterogeneity of Vaginal Microbial Communities within Individuals". Journal of Clinical Microbiology 47, 1181-1189.

Kim W, Flamm SL, Di Bisceglie AM and Bodenheimer HC 2008. Serum activity of alanine amino transferase (ALT) as an indicator of health and disease. Hepatology 47, 1363-1370.

Knap PW 2005. Breeding robust pigs. Australian Journal of Experimental Agriculture 45, 763-773.

Koenig G and Seneff S 2015. Gamma-glutamyl transferase: a predictive biomarker of cellular antioxidant in adequacy and disease risk. Dis Markers 818570.

Kostic AD, Howitt MR and Garrett WS 2013. Exploring host–microbiota interactions in animal models and humans. Genes Dev 27, 701-718.

Kraimi N, Dawkins M, Gebhardt-Henrich SG, Velge P, Rychlik I, Volf J, Creach P, Smith A, Colles F and Leterrier C 2019. Influence of the microbiota-gut-brain axis on behavior and welfare in farm animals: A review. Physiology & Behavior 210: 112658.

Kunze FA and Hottiger MO 2019. Regulating Immunity via ADP-Ribosylation: Therapeutic Implications and Beyond. Trends in Immunology 40, 159-173.

Lafuente E and Beldade P 2019. Genomics of Developmental Plasticity in Animals. Front Genet 10: 720.

Langer L, Gabor E, Liebeton K, Meurer G, Niehaus F, Schulze R, Eck J and Lorenz P 2006. Metagenomics: An inexhaustible access to nature's diversity. Biotechnology Journal 1, 815-821.

Langille MGI, Zaneveld J, Caporaso G, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Thurber RLV, Rob Knight R, Robert G, Beiko RG and Huttenhower C 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 31, 814-821.

Langmead B and Salzberg SL 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357-359.

Larzul C, Le Roy P, Tribout T, Gogue J and SanCristobal M 2006. Canalyzing selection on ultimate pH in pigs: consequences on meat quality. Porc 8th WCGALP, August 13-18, Belo Horizonte, Brasil, 4p.

Leineweber C, Müller E and Marschang RE 2018. Blood reference intervals for rabbits (Oryctolagus cuniculus) from routine diagnostic samples. Bestimmung von Blutreferenzwerten für Kaninchen (Oryctolagus cuniculus) aus Routine diagnostic proben. Tierarztl Prax Ausg K Kleintiere Heimtiere 46, 393-398.

Lepage P, Hasler R, Spehlmann ME, Rehman A, Zvirbliene A, Begun A, Ott S, Kupcinskas L, Doré J, Raedler A and Schreiber S 2011. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. Gastroenterology 141, 227-236.

Ley RE, Peterson DA and Gordon JI 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124, 837–848.

Lidfors L and EdströmT 2010. The Laboratory Rabbit. In The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals, 8th ed.; Hubrecht, R., Kirkwood, J., Eds. Wiley-Blackwell: West Sussex, UK 399-417.

Liu YA, Wang YX and Jiang CL 2017. Inflammation: The Common Pathway of Stress-Related. Diseases 11, 316-327.

Liu YZ, Wang YX and Jiang CL 2017. Inflammation: The Common Pathway of Stress-Related Diseases. Front Hum Neurosci 11, 316-327.

Lockwood KM, Marsland AL, Cohen M and Gianaros PJ 2016. Sex Differences in the association between stressor-evoked interleukin-6 reactivity and C-reactive protein. Brain. Behav. Immun 58, 173-180.

Lung LHDS, Carvalheiro R, Neves HHDR and Mulder HA 2020. Genetics and genomics of uniformity and resilience in livestock and aquaculture species: A review. J Anim Breed Genet 137, 263-280.

Ma TX and Gao Y 2020. New insights into roles of basophils in initiating T helper type 2 immunity. Chinese Herbal Medicines 12, 14-18.

Maccaferri S, Biagi E and Brigidi P 2011. Metagenomics: key to human gut microbiota. Dig Dis 29, 525-30.

Magoč T and Salzberg SL 2011. FLASH*:* fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957-2963.

Mainau E and Manteca X 2011 Pain and discomfort caused by parturition in cows and sows. Appl. Anim. Behav. Sci 135, 241-251.

Mahgoub O, Kadim IT, Tageldin MH, Al-Marzooqi WS, Khalaf SQ and Ali AA 2008. Clinical profile of sheep fed non-conventional feeds containing phenols and condensed tannins. Small Ruminant Research 78, 115-122.

Markanday A 2015. Acute phase reactants in infections: evidence-based review and a guide for clinicians. Open Forum Infectious Diseases 2, 1-7.

Martín-Fernández JA, Barceló-Vidal C and Pawlowsky-Glahn V 2000. Zero Remplacement in Compositional Data Sets. In: Kiers HAL, Rasson JP, Groenen PJF, Schader M (eds) Data Analysis, Classification, and Related Methods. Studies in Classification, Data Analysis, and Knowledge Organization. Springer, Berlin, Heidelberg.

Martín-Fernández JA, Hron K, Templ M, Filzmoser P and Palarea-Albaladejo J 2015. Bayesian-multiplicative treatment of count zerosin compositional data sets. Stat. Modelling 15, 134-158.

Martin WJ 1989. New technologies for large-genome sequencing. Genome 31, 1073-1080.

Masaki T and Yoshimatsu H 2006. The hypothalamic H1 receptor: a novel therapeutic target for disrupting diurnal feeding rhythm and obesity. Trends Pharmacol Sci 27, 279-284.

Mbanasor UU, Anene BM, Chinezie AB, Nnaji TO, Eze JI and Ezekwe AG 2003. Haematology of normal trypanosome infected Muturu cattle in southeastern Nigeria. Nigerian Jour. Anim. Prod 30, 236-241.

Metzker ML 2010. Sequencing technologies — the next generation. Nature Reviews Genetics 11, 31-46.

Michelland RJ, Combes S, Monteils V, Cauquil L, Gidenne T and Fortun-Lamothe L 2011. Rapid adaptation of the bacterial community in the growing rabbit caecum after a change in dietary fibre supply. Animal 5, 1761-1768.

Moore MD, Zimmerman K and Smith AS 2015.Hematologicalassessment in pet rabbits blood sample collection and blood cell identification. Vet. Clin. North. Am. Exot. Anim. Pract18, 9-19.

Mormede P, Boisseau-Sowinski L, Chiron J, Diederich C, Eddison J, Guichet JL, Le Neidre P and Meunier-Salaun MC 2018. Bien-être animal: context, definition, évaluation. INRA Prod. Anim 31, 145-162.

Moynagh PN 2005. TLR signalling and activation of IRFs: revisiting old friends from the NFkappaB pathway. Trends Immunol 26, 469-476.

Mulder HA, Hill WG, Vereijken A and Veerkamp RF 2009. Estimation of genetic variation in residual variance in female and male broilers. Animal 3, 1673-1680.

Mulder HA, Hill WG and Knol EF 2015. Heritable environmental variance causes nonlinear relationships between traits: application to birth weight and stillbirth of pigs. Genetics 199, 1255-1269.

Mulder HA and Rashidi H 2017. Selection on resilience improves disease resistance and tolerance to infections. Journal of Animal Science 95, 3346-3358.

Ostman S, Rask C, Wold AE, Hultkrantz S and Telemo E 2006. Impaired regulatory T cell function in germ-free mice. Eur J Immunol 36, 2336-2346.

Otero RW, Velasco H and Sandoval H 2009. The protective role of bilirubin in human beings. Revista Colombiana de Gastroenterología 24, 293–301.

Ouwehand A and Vesterlund S 2003. Health aspects of probiotics. IDrugs 6, 573-580.

Palarea-Albaladejo J and Martín-Fernández JA 2015. zCompositions – R package for multivariate imputation of left-censored data under a compositional approach. Chemom. Intell. Lab. Syst 143, 85-96.

Pegg AE 2013. Toxicity of polyamines and their metabolic products. Chem Res Toxicol 26, 1782-1800.

Petta I, Fraussen J, Somers V and Kleinewietfeld M 2018. Interrelation of Diet, Gut Microbiome, and Autoantibody Production. Front Immunol 9: 439.

Rauw WM 2012. Immune response from a resource allocation perspective. Front. Genet 3, 267.

Read T, Fortun-Lamothe L, Pascal G, Le Boulch M, Cauquil L, Gabinaud B, Bannelier C, Balmisse E, Destombes N, Bouchez O, Gidenne T and Combes S 2019. Diversity and Co-occurrence Pattern Analysis of Cecal Microbiota Establishment at the Onset of Solid Feeding in Young Rabbits. Front Microbiol 10: 973.

Reyes A, Haynes M, Hanson N, Angly FE, Heath AC, Rohwer F and Gordon JI 2010. Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature 466, 334-338.

Robles Alonso V and Guarner F 2013. Linking the Gut Microbiota to Human Health. Br J Nutr 109, 21-26.

Roesch LFW, Dobbler PT, Pylro VS, Kolaczkowski B, Drew JC and Triplett EW 2019. PIME: A package for discovery of novel differences among microbial communities. Mol Ecol Resour 20, 415-428.

Rönnegård L, Felleki M, Fikse WF, Mulder H and Strandberg E 2013. Variance component and breeding value estimation for genetic heterogeneity of residual variance in Swedish Holstein dairy cattle. Journal of Dairy Science 96, 2627-2636.

Round JL and Mazmanian SK 2009. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 9, 313-323.

SanCristobal-Gaudy M, Elsen JM, Bodin L and Chevalet C 1998. Prediction of the response to a selection for canalisation of a continuous trait in animal breeding. Genetics Selection Evolution 30, 423-451.

Scheffer M, Carpenter SR, Dakos V and Van Nes EH 2015. Generic indicators of ecological resilience: inferring the chance of a critical transition. Annu. Rev. Ecol. Evol. Syst 46, 145-167.

Schmieder R and Edwards R 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27, 863-864.

Schueller, K, Riva A, Pfeiffer S, Berry D and Somoza V 2017. Members of the Oral Microbiota Are Associated with IL-8 Release by Gingival Epithelial Cells in Healthy Individuals. Frontiers in Microbiology 8, 416-425.

Scrivo R, Vasile M, Bartosiewicz I and Valesini G 2011. Inflammation as “common soil” of the multifactorial diseases. Auto immun Rev 10, 369-374.

Sekirov I, Russell SL, Antunes LC and Finlay BB 2010. Gut microbiota in health and disease. Physiol Rev 90, 859-904.

Sell-Kubiak E, Bijma P, Knol EF and Mulder HA 2015. Comparison of methods to studyuniformity of traits: application to birth weight in pigs. Journal of Animal Science 93, 900-911.

Sender R, Fuchs S and Milo R 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLOS Biology 14: e1002533.

Sharma A, Tate M, Mathew G, Vince JE, Ritchie RH and De Han JB 2018. Oxidative Stress and NLRP3-Inflammasome Activity as Significant Drivers of Diabetic Cardiovascular Complications: Therapeutic Implications. Front. Physiol 9: 114.

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN and Garrett WS 2013. The microbial metabolites, shortchain fatty acids, regulate colonic Treg cell homeostasis. Science 341, 569-573.

Sorensen D and Waagepetersen R 2003. Normal linear models with genetically structured residual variance: a case study. Genetics Research 82, 202-222.

Spor A, Koren O and Ley R 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. Nature Rev. Microbiol 9, 279-290.

Stilwell G, Carvalho RC, Lima MS and Broom DM 2009. Effect of caustic paste disbudding, using local anaesthesia with and without analgesia, on behaviour and cortisol of calves. Appl. Anim. Behav. Sci 116, 35-44.

Stoner L, Lucero AA, Palmer RB, Jones ML, Young MJ and Faulkner J 2013. Inflammatory biomarkers for predicting cardiovascular disease. Clinical Biochemistry 46, 1353-1371.

Streit W and Schmitz R 2004. Metagenomics – the key to the uncultured microbes. Current Opinion in Microbiology 7, 492-498.

Tamames J and Puente-sánchez F 2019. SqueezeMeta , A Highly Portable , Fully Automatic Metagenomic Analysis Pipeline 9, 1–10.

Tanaka T, Narazaki M and Kishimoto T 2014. IL-6 in inflammation, immunity, and disease. Cold Spring Harb. Perspect. Biol 6: a016295.

Thomas T, Gilbert J and Meyer F 2012. Metagenomics - a guide from sampling to data analysis. Microb Inform Exp 2: 3.

Varmuza K and Filzmoser P 2009. Introduction to Multivariate Statistical Analysis in Chemometrics. CRC Press, Taylor & Francis Group.

Velasco-Galilea M, Piles M, Viñas M, Rafel O, González-Rodríguez O, Guivernau M and Sánchez JP 2018. Rabbit Microbiota Changes Throughout the Intestinal Tract. Front Microbiol 9, 2144-2157.

Washington IM and Van Hoosier G 2012. Clinical Biochemistry and Hematology. In The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents. Suckow, M.A., Stevens K.A., Wilson R.P. Eds: Elsevier 57–116.

Webster JL, TonelliL and Sternberg EM 2002. Neuroendocrine regulation of immunity. Ann Rev Immunol 20, 125-163.

Wilson M 2018. The Human Microbiota in Health and Disease: An Ecological and Community. Garland Science 505.

World Organisation for Animal Health 2014. Terrestrial Animal Health Code. Chapter 7.1. Availableat http://www.oie.int/en/international-standard-setting/terrestrial-code/access online/.

Wu WM, Yang YS and Peng LH 2014. Microbiota in the Stomach: New Insights. Journal of Digestive Diseases 15, 54-61.

Yang Y, Christensen OF and Sorensen D 2011. Analysis of a genetically structured varianceheterogeneity model using the Box-Cox transformation. Genetics Research 93, 33-46.

Zang B and Powers R 2012. Analysis of bacterial biofilms using NMR-based metabolomics. Future Med Chem 4, 1273-1306.

Zhang JM, and An J 2007. Cytokines, inflammation, and pain. Int. Anesthesiol. Clin 45, 27-37.

Zhao X, Li L, Li X, Li J, Wang D and Zhang H 2019. The relationship between serum bilirubin and inflammatory bowel disease. Hindawi: Mediators of Inflammation: 5256460.