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ORIGINAL ARTICLE

Genome-wide association studies for diarrhoea outcomes identified genomic regions affecting resistance to a severe enteropathy in suckling rabbits

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Abstract

Selection and breeding strategies to improve resistance to enteropathies are essential to reaching the sustainability of the rabbit production systems. However, disease heterogeneity (having only as major visible symptom diarrhoea) and low disease heritability are two barriers for the implementation of these strategies. Diarrhoea condition can affect rabbits at different life stages, starting from the suckling period, with large negative economic impacts. In this study, from a commercial population of suckling rabbits (derived from 133 litters) that experienced an outbreak of enteropathy, we first selected a few animals that died with severe symptoms of diarrhoea and characterized their microbiota, using 16S rRNA gene sequencing data. *Clostridium* genus was consistently present in all affected specimens. In addition, with the aim to identify genetic markers in the rabbit genome that could be used as selection tools, we performed genome-wide association studies for symptoms of diarrhoea in the same commercial rabbit population. These studies were also complemented with F_{ST} analyses between the same groups of rabbits. A total of 332 suckling rabbits (151 with severe symptoms of diarrhoea, 42 with mild symptoms and 129 without any symptoms till the weaning period), derived from 45 different litters (a subset of the 133 litters) were genotyped with the Affymetrix Axiom OrcunSNP Array. In both genomic approaches, rabbits within litters were paired to constitute two groups (susceptible and resistant, including the mildly affected in one or the other group) and run case and control genome-wide association analyses. Genomic heritability estimated in the designed experimental structure integrated in a commercial breeding scheme was 0.19–0.21 (s.e. 0.09–0.10). A total of eight genomic regions on rabbit chromosome 2 (OCU2), OCU3, OCU7, OCU12, OCU13, OCU16 and in an unassembled scaffold had significant single nucleotide polymorphisms (SNPs) and/or markers that trespassed the F_{ST} percentile distribution. Among these regions, three main peaks of SNPs were identified on OCU12, OCU13 and OCU16. The QTL region on OCU13 encompasses

several genes that encode members of a family of immunoglobulin Fc receptors (*FCER1G*, *FCRLA*, *FCRLB* and *FCGR2A*) involved in the immune innate system, which might be important candidate genes for this pathogenic condition. The results obtained in this study demonstrated that resistance to an enteropathy occurring in suckling rabbits is in part genetically determined and can be dissected at the genomic level, providing DNA markers that could be used in breeding programmes to increase resistance to enteropathies in meat rabbits.

KEYWORDS

candidate gene, disease resistance, immunoglobulin fc receptor, innate immunity, microbiota, *Oryctolagus cuniculus*, SNP

1 | INTRODUCTION

Selection and breeding strategies to improve disease resistance and the related components, resilience and tolerance (Bishop & Woolliams, 2014; Knap & Doeschl-Wilson, 2020), are essential to reaching sustainability of the animal production systems. Genetic progress for these objectives can also lead to reduced use of antibiotics, improved animal welfare, reduced mortality and, in turn, direct and indirect reduced production costs, which might also contribute to reduce the overall carbon footprint of the livestock sector (Gunia et al., 2018; Phocas et al., 2016). However, in practice, the implementation of selection strategies to improve disease resistance is quite complex for several reasons, including, the facts that (i) many different diseases/pathogens might be relevant or involved, each with its own biological and epidemiological peculiarities, (ii) it is difficult or economically not feasible to record and collect useful phenotypes/information for a comprehensive genetic evaluation, (iii) even in cases where this would be feasible, heritability of relevant traits is usually very low, and (iv) environments and natural challenging conditions of commercial farms are usually very different from those of the selection nuclei, making the transfer of the genetic progress, from the selection nuclei to the basis of the pyramidal animal production structure, not completely effective.

Enteropathies and related digestive disorders are among the main causes of economic losses in rabbitries. These enteric problems, whose main clinical sign is diarrhoea, are relevant both in suckling and post-weaning growing rabbits; on average, diarrhoea may lead to an overall mortality of about 8%–10%, reaching, in some conditions, even 50% or higher levels (Harcourt-Brown, 2002; Licois et al., 2005, 2006; Marlier et al., 2003; Rashwan & Marai, 2000; Rosell, 2003; Solans et al., 2019). Enteropathies are determined by several pathogenic factors, including an imbalanced fibre diet, coccidiosis, epizootic enterocolitis from a few bacterial and viral infections and co-infections that may vary in frequency and co-occurrence, making very difficult to disentangle the complexity and the causative agents of this multi-factorial and heterogeneous pathogenic condition in rabbits (Garreau et al., 2006, 2021; Harcourt-Brown, 2002; Lavazza et al., 2008). In more details, several pathogens have been reported to be the causative or co-causative agents of enteric diseases in rabbits, including enteropathogenic *Escherichia coli* (ETEC) strains (considered among the most frequent agents, also at the pre-weaning phase), *Clostridium spiroforme*, *C. perfringens*, *Enterococcus hirae*, *Enterobacter sakazakii, Barceroides fragilis*, *Akkermansia muciniphila*, *Salmonella* spp., several coccidian protozoa of the *Eimeria* genus and group A rotavirus (Agnoletti et al., 1999; Carman & Borriello, 1984; Djukovic et al., 2018; Dow et al., 2005; Garcia et al., 2014; Jin et al., 2018; Lavazza et al., 2008; Malo, 2019; Myers et al., 1989; Solans et al., 2019; Vela et al., 2010).

Resistance to this complex and heterogeneous pathogenic condition and related infections in rabbits is in part genetically determined, with estimated heritability that ranges from 0.02 to 0.08 using simple records of disease syndrome, which may vary according to the heterogeneity of the potential causative agents, growing phase and the co-effects of nutritional, maternal and other environmental factors (De Rochambeau et al., 2006; Eady et al., 2007; García-Quirós et al., 2014; Garreau et al., 2006, 2008; Gunia et al., 2015; Licois et al., 2005). This evidence opened the possibility for the implementation of selection strategies to increase resistance against enteropathies and related pathogenic conditions in commercial meat rabbit populations and investigate the genetic factors involved in this complex pathogenic status (Garreau et al., 2008, 2012, 2021). Several studies reported that polymorphisms in candidate genes involved in host immunity might be associated with the susceptibility of non-specific digestive disorders in growing rabbits of a few lines (Chen, Zhang, et al., 2013; Fu et al., 2014, 2015; Li et al., 2018; Liu et al., 2013, 2017; Yang et al., 2013; Zhang et al., 2011;

Zhang, Zhang, Chen, et al., 2013; Zhang, Zhang, Peng, et al., 2013). However, it seems clear that the association reported in these investigations should be validated and confirmed with other studies that can provide more comprehensive genome-wide analyses, in different lines and in specifically defined environments, which could also assure that all involved animals have been in contact with the pathogenic agents or conditions. In addition, most of these studies have involved growing rabbits and it seems clear that different production phases (suckling, weaning and growing) might have to face different types of potential enteropathies. Therefore, mainly due to the heterogeneity and complexity of these types of disorders in rabbits and, in turn, the complications of the experimental procedures that should be designed, none of the studies that thus far investigated, at the rabbit genome level, DNA markers associated with this pathogenic condition, also attempted to obtain a precise characterization of the putative biological components (e.g., microbiota, viroma) involved as causative factors or derived consequence of the disorder. Only few studies reported information on the microbiota characterization of diarrhoea in rabbits linked to different feeding strategies in specific experimental designs (Chen et al., 2022; Wang, Fan, et al., 2022).

A few genome-wide association studies (GWAS) for several production traits have been recently carried out in rabbit after, also for this livestock species, genomic tools, including a single nucleotide polymorphism (SNP) array and a reference genome, have been made available to the scientific community (Bovo et al., 2021; Casto-Rebollo et al., 2020; Fontanesi et al., 2021; Laghouaouta et al., 2020; Liao et al., 2021; Mora et al., 2022; Sánchez et al., 2020; Sosa-Madrid, Hernández, et al., 2020; Sosa-Madrid, Santacreu, et al., 2020; Wang, Xie, et al., 2022; Yang et al., 2020). However, thus far, no GWAS have been carried out in this livestock species to identify DNA markers associated with resistance to any enteropathies.

In this study, with the aim to identify regions of the rabbit genome associated with resistance to a severe enteropathy of sucking rabbits, that occurred in a commercial selection husbandry system, we designed a balanced case and control genome-wide association study, integrated with F_{ST} analyses.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Animal samples used in this study were collected following the recommendation of directive 2010/632.1. Animals were not raised or treated in any way for this study, and samples were collected by the farmers during the routine inspection of the animals. Only from naturally dead animals tissue samples were collected. The study has been conducted in a commercial meat rabbitry that followed standard husbandry procedures and approved veterinary practices.

2.2 | Animals

This study included a population of 1016 suckling rabbits from 133 litters (obtained by crossing eight bucks with 136 does; only one buck per doe), each constituted by 6–8 suckling rabbits. Does did not receive any antibiotic treatment. Rabbits were from a nucleus of a commercial line, that is under selection to improve growth rate. All these rabbits were raised in the same farm (located in the North of Italy), in the same building and all litters were contemporaneous (obtained over 2 months, from March to April). Among these litters, a total of 45 litters were then chosen because they had both healthy suckling rabbits (without any external symptom of diarrhoea overall the suckling period) and diseased suckling rabbits (with very evident symptoms of diarrhoea before weaning), as shown in Figure 1. This within-litter disease-based classification of the suckling rabbits made possible to pair within-litter animals to control stratification and environmental factors in a case and control experimental design that included the following classes of phenotyped suckling rabbits: (i) completely healthy rabbits (n. 129), that were animals that reached the weaning age (30–35 days) without any symptoms of diarrhoea (2–4 rabbits for each litter), and considered to be resistant to diarrhoea; (ii) rabbits with severe symptoms of diarrhoea (n. 151) that did not reach the weaning age as they died before, due to this enteric problem (2–4 rabbits per litters), and considered to be susceptible to diarrhoea. In the classification of the suckling rabbits, in some of the same litters, we also identified animals (n. 42) that showed mild symptoms of diarrhoea in the suckling phase and that however reached the weaning age (0–2 within each of the 45 chosen litters).

2.3 | 16S rRNA gene sequencing of diarrhoea samples and bioinformatic analyses

The rectal content of five suckling rabbits of different litters (RAB1-5), that died from severe diarrhoea, was collected, and total DNA was extracted using the QIAamp DNA mini kit (Qiagen), following manufacturer's instructions. DNA was quality checked in a TBE 1% agarose gel after staining with 1× GelRed Nucleic Acid Gel Stain (Biotium Inc).

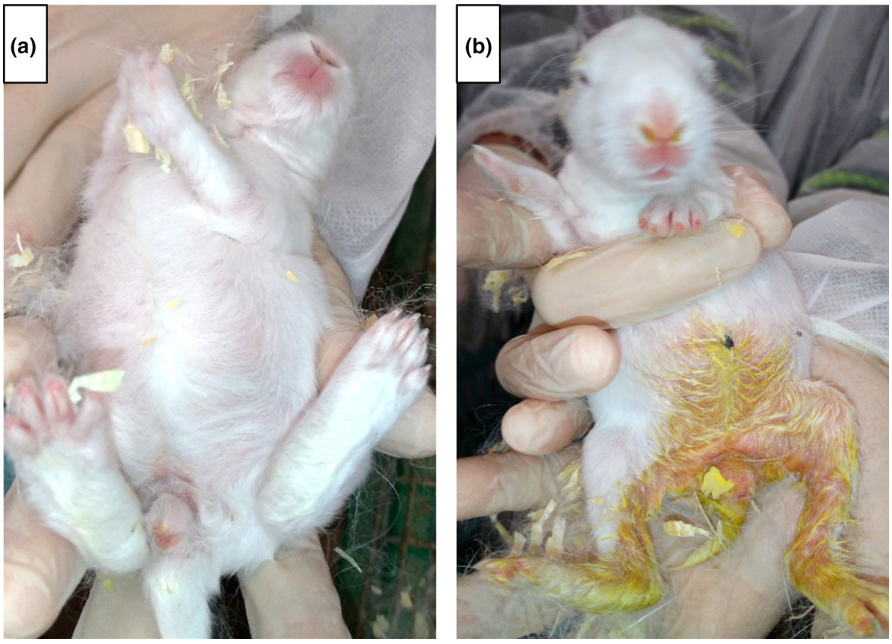

FIGURE 1 Extreme phenotypes of the pre-weaned rabbits considered in the study. (a) A resistant rabbit (without any external symptoms of diarrhoea overall the pre-weaning period); (b) a highly susceptible rabbit (with very evident external symptoms of diarrhoea before weaning). [Colour figure can be viewed at wileyonlinelibrary.com]

DNA concentration was measured using a Qubit 4.0 fluorimeter (Thermo Fisher Scientific). The bacterial 16S rRNA was amplified with the universal primer pairs and PCR conditions reported by Huse et al. (2008), by targeting the V3 region of the 16S gene: 338F: 5'-ACTCCTAC GGGAGGCAGCAG-3′ and 533R: 5'-TTACCGCGGCT GCTGGCAC-3′. Amplifications were performed on a SimpliAmp Thermal Cycler (Thermo Fisher Scientific). Reactions were run in a total volume of 20 μL including KAPA HiFi HotStart Mastermix (Roche); 10 pmol of each primer; 40 ng of template DNA. Amplicons were quality checked in a TBE 2.5% agarose gel after staining with 1× GelRed Nucleic Acid Gel Stain (Biotium Inc.) and purified using standard isopropanol alcohol precipitation protocol. Libraries were prepared as previously described (Bovo et al., 2020). Sequencing of amplicons was carried out on an Ion S5-Ion Chef System (Thermo Fisher Scientific Inc).

Sequenced reads underwent to an initial quality check with the Torrent Suite v.5.8.0 (Thermo Fisher Scientific Inc). Then, only reads presenting both primers at the DNA ends were processed with PRINSEQ Lite v.0.20.4 (Schmieder & Edwards, 2011) as follows: (i) trimming of the 5′- and 3′-ends up to reaching a base with a $Q > 20$, (ii) exclusion of reads with a size <20 bp and (iii) exclusion of reads with an average $Q < 20$. Taxonomic assignment was based on the Bacteria 16S RefSeq collection v.07/2023 (O'Leary et al., 2016) and the NCBI Taxonomy (Schoch et al., 2020). Reads were mapped over the 16S DNA database with BLAST + v.2.7.1 (algorithm blastn, default parameters) (Camacho et al., 2009) and taxonomic assignment followed the Lowest Common Ancestor (LCA) approach. We considered as reasonably annotated reads having the following alignment scores: E-value ≤0.00001, sequence coverage ≥95% and a sequence identity ≥95%. At least two reads were required to support the presence of a given bacteria. Sequencing data were evaluated by means of rarefaction curves as described by Bovo et al. (2020).

2.4 | Genotyping of the investigated rabbits

DNA of a total of 322 suckling rabbits (derived by summing up all rabbits of the 45 litters that were classified according to the absence and presence, and level of severity, of external symptoms of diarrhoea) was extracted from hair or ear tissue using the Wizard® Genomic DNA Purification kit (Promega Corporation). Animals were genotyped with the Affymetrix Axiom OrcunSNP Array (Affymetrix Inc.), which genotypes a total of 199,692 DNA markers, following the manufacturer's procedures. BLAST+ v.2.7.1 (Camacho et al., 2009) was used to map DNA markers to the OryCun2.0 reference genome (GCA_000003625.1). Markers assigned to more than one position or assigned to sex chromosomes were discarded. Quality control and data filtering were carried out with the Axiom™ Analysis Suite, by retaining only markers located in autosomes and scaffolds, labelled as best recommended, presenting clearly separated genotype clusters, a minor allele frequency (MAF) > 0.05 and that were in Hardy–Weinberg equilibrium ($p > 0.0001$). A total of 45,404 very high-quality DNA markers were retained.

2.5 | Genome-wide association analyses

To take advantage from the extreme phenotypes related to resistance/susceptibility to diarrhoea observed within

litter (i.e., healthy suckling rabbits for the whole suckling period without any external symptoms of diarrhoea, considered to be resistant; suckling rabbits with heavy external signs of diarrhoea leading to death, considered to be susceptible), different genome-wide association analyses based on a case and control design were carried out. As reported in Table 1, these included: (i) GWAS1, that is the contrast between the two extreme groups (129 vs 151 rabbits), (ii) GWAS2, that is the contrast between a group constituted by merging severely affected rabbits with mildly affected rabbits (n. 151 + 42) against resistant rabbits (n. 129) and (iii) GWAS3, that is the contrast between a group constituted by merging resistant rabbits with mildly affected rabbits (n. 129 + 42) against severely affected rabbits (n. 151). The following general linear mixed model was used:

$$y = x\beta + g + e$$

where y $(n \times 1)$ is a vector containing the phenotype (as defined in the three scenarios described above: 0, controls = resistant to diarrhoea; 1, cases = susceptible to diarrhoea) for the n^{th} animal, x $(n \times 1)$ is the vector containing genotypes for the i^{th} DNA marker, β is the additive fixed effect of the i^{th} DNA marker on the phenotype, $g \sim N(0,\sigma^2_g K)$ is a multivariate Gaussian polygenic effect, with covariance matrix proportional to the centred genomic relatedness matrix K $(n \times n)$ and $e \sim N(0,\sigma^2_e I)$ is a multivariate Gaussian vector of uncorrelated residuals. The assessment of the association was obtained by testing the null hypothesis $H_0:\beta = 0$ (Wald test). Models were fitted with GEMMA v. 0.98 (Zhou & Stephens, 2012). The Bonferroni corrected significance level threshold equal to a nominal value of 0.05 was used to define significant markers. A marker was declared suggestively associated with the phenotype if it had a $p < 5.0 \times 10^{-05}$. GEMMA was used to estimate the genomic heritability (h^2_G). Genomic control inflation factors (λ_{GC}), Quantile-Quantile plots (QQ plots) and Manhattan plots were generated in R v.4.2.2 (R Core Team, 2022).

2.6 | F_{ST} analyses

Wright's Fixation Index (F_{ST}) was calculated for each SNPs in the pairwise comparison between the resistant group of suckling rabbits and the susceptible group of suckling rabbits (with the same combinations defined above for the GWAS analyses: $F_{ST}1$, $F_{ST}2$ and $F_{ST}3$). F_{ST} values were computed in PLINK 1.9 (Chang et al., 2015) that implements the Weir and Cockerham (1984) method. We considered as outliers those markers having a F_{ST} value equal or above the 99.98th percentile of the related distribution (top 11 SNPs). Manhattan plots were generated in R v.4.2.2 (R Core Team, 2022).

2.7 | Gene annotation and haploblock analysis

Annotated genes close to relevant DNA markers (±500 kb flanking regions) were retrieved from the OryCun2.0 NCBI's GFF file (NCBI *Oryctolagus cuniculus* Annotation Release 102). The functional relevance of the genes was evaluated based on a detailed analysis of the scientific literature and Gene Cards information (Stelzer et al., 2016). Enrichr (Chen, Tan, et al., 2013) was used in the gene enrichment analysis that was performed with the following databases: GWAS Catalog 2023 (https://www.ebi.ac.uk/gwas/), KEGG Human database 2021 (KEGG, http://www.kegg.jp/), MGI mammalian phenotype level 4 (https://www.informatics.jax.org/vocab/mp_ontology), and the biological process branch of gene ontology (GO:BP 2023; http://geneontology.org/). Enrichr input was the whole set of genes ($n = 69$) located ±500 kb from the top markers identified with different methods and reported in Table 2. We considered statistically enriched terms having: (i) at least two genes of the input set related to at least two different genome regions and (ii) an adjusted $p < 0.05$.

Linkage disequilibrium (LD) was studied with Haploview (Barrett et al., 2005) using default parameters.

TABLE 1 Details of the genome-wide association studies (GWAS) for diarrhoea in the suckling rabbits.

GWAS approach	Groups of rabbits	No. of animals	λ_{GC}[a]	h^2_G (s.e.)[b]
GWAS1	Severe symptoms of diarrhoea vs. unaffected (without any symptoms of diarrhoea)	151 + 129 (280)	1.04	0.21 (0.10)
GWAS2	Affected (severe symptoms + mild symptoms of diarrhoea) vs. unaffected (without any symptoms of diarrhoea)	193 + 129 (322)	1.02	0.19 (0.10)
GWAS3	Severe symptoms of diarrhoea vs. (unaffected + mild symptoms of diarrhoea)	151 + 171 (322)	1.04	0.19 (0.10)

[a]Genomic control inflation factor.

[b]Genomic heritability estimated from SNP data. The standard error is reported in parentheses.

TABLE 2 Summarized results obtained combining the genome-wide association studies (GWAS1-3) and F_{ST} analyses (F_{ST}1-3).

Analysis[a]	OCU[b]	Marker	Position	Mi/Ma[c]	Pop[d]	R[e]	S[f]	β[g]	P[h]	F_{ST} value[i]	Genes
GWAS1, GWAS3, $F_{ST}1$, $F_{ST}3$	2	AX-147110011	166,302,108	G/A	0.07	0.13	0.02	−0.44	$*3.35\times10^{-08}$	§0.076	*RAD51AP2, SMC6, VSNL1, GEN1*
GWAS1, GWAS3, $F_{ST}1$	3	AX-147151286	140,492,290	G/A	0.08	0.13	0.03	−0.45	$*4.39\times10^{-08}$	§0.068	*TRMT12, KIAA0196, NDUFB9, LOC100353158, TATDN1, TMEM65, ZNF572, MTSS1, NSMCE2, RNF139, SQLE*
GWAS1, GWAS3	7	AX-147178054	162,786,466	T/C	0.06	0.11	0.02	−0.41	$*9.05\times10^{-07}$	-	*EPHA4*
GWAS1, GWAS3, $F_{ST}1$, $F_{ST}2$	12	AX-147124755	29,704,696	G/A	0.30	0.40	0.23	−0.28	$§4.64\times10^{-07}$	§0.064	*KIF6, LRFN2, MOCS1, DAAM2*
GWAS1, GWAS3, $F_{ST}1$, $F_{ST}2$	12	AX-147169968	30,469,270	C/A	0.23	0.33	0.16	−0.30	$§4.95\times10^{-07}$	§0.072	*OARD1, LRFN2, LOC100348238, UNC5CL, TREML2, APOBEC2, NFYA, TREML1, LOC100348748, TSPO2*
GWAS1, GWAS3, $F_{ST}1$, $F_{ST}3$	13	AX-147129897	31,348,345	C/T	0.08	0.14	0.03	−0.41	$*1.42\times10^{-07}$	*0.082	*LOC100337909, TOMM40L, HSPA6, UFC1, CFAP126, PPOX, LOC100356569, USP21, ADAMTS4, FCER1G, PCP4L1, APOA2, B4GALT3, FCRLA, FCRLB, LOC100359306, FCGR2A, LOC100338913, DEDD, DUSP12, OLFML2B, NR1I3, NDUFS2, SDHC, ATF6, MPZ*
(GWAS2), $F_{ST}2$	16	AX-147054369	67,591,685	G/A	0.19	0.27	0.14	−0.25	1.91×10^{-06}	0.054	*KISS1, ZC3H11A, PLEKHA6, NFASC, SOX13, LRRN2, PIK3C2B, GOLT1A, PPP1R15B, REN, ETNK2, MDM4, SNRPE*
GWAS1, GWAS3, $F_{ST}1$, $F_{ST}3$	NW_003160554.1	AX-147143772	18,430	G/A	0.35	0.45	0.26	−0.28	$*9.50\times10^{-09}$	*0.092	-

Note: Only the top markers in the region ±500 kb (and related annotation) are reported. All results are reported in Tables S5 and S6.

[a]GWAS1/$F_{ST}1$: severely affected rabbits against the unaffected rabbits; GWAS2/$F_{ST}2$: affected rabbits (severe and mild diarrhoea) against the unaffected rabbits; GWAS3/$F_{ST}3$: severely affected rabbits against resistant (unaffected and mild diarrhoea) rabbits. In genome-wide association studies, top significant markers and top suggestively significant markers (within brackets) are listed for the corresponding genome regions also identified in the F_{ST} analyses.

[b]Rabbit chromosome.

[c]Minor/Major alleles.

[d]Frequency of the minor allele in the considered rabbit population (322 rabbits).

[e]Frequency of the minor allele in the resistant rabbits.

[f]Frequency of the minor allele in the susceptible rabbits.

[g]Regression coefficient of the genome-wide association studies: when information was confirmed by GWAS1 and GWAS3, regression coefficient was reported for the most significant analysis. A negative value indicates that the minor allele is associated with disease resistance.

[h]P at the Wald test (GEMMA) for the most significant analysis in the genome-wide association studies (§: GWAS1; *: GWAS3).

[i]The highest F_{ST} value was reported (§: $F_{ST}1$; *: $F_{ST}3$).

3 | RESULTS

3.1 | Frequency of diarrhoea in the monitored suckling rabbit population

Among the 133 monitored litters, accounting a total of 1016 suckling rabbits, on average three rabbits per litter were healthy (without any visible external symptoms of diarrhoea over all the suckling period; Figure 1) and five rabbits per litter had signs of diarrhoea. A total of 74 litters (54%) had 100% of severely affected suckling rabbits with clear signs of diarrhoea (Figure 1), that died before reaching the weaning age due to this pathogenic condition (100% mortality). Fifteen litters (11%) did not have any sick animals (animals without any evident visible external symptoms of diarrhoea till weaning) whereas the remaining 47 litters (35%) had at least one affected rabbit. Considering the whole population, the distribution of the percentage of affected suckling rabbits per litter is given in Figure S1.

We then identified a total of 45 litters that had each a balanced number of severely affected animals (that died before weaning) together with a similar number of unaffected individuals (for a total of 151 and 129 rabbits, respectively). All suckling rabbits within the same litter shared the same mother and environmental conditions, which may be the source of shared pathogens or pathogenic elements that would trigger diarrhoea in the susceptible animals. In some of these litters, we also noted the presence of a total of 42 additional animals that had mild symptoms of diarrhoea (i.e., with an intermediate phenotype), which did not prevent them to reach the weaning age.

3.2 | Characterization of the diarrhoea microbiota

To obtain a first characterization of the microbiota involved in the pathogenic condition or that could be the result of this pathogenic state, we analysed the rectal content of five suckling rabbits of different litters (RAB1-5) that died before weaning and that had severe signs of diarrhoea. About 371,000–552,000 high-quality reads per sample were obtained (Table S1). On average, taxonomic assignment was successful for 97% of the sequenced reads despite the stringent alignment parameters (sequence coverage ≥95% and a sequence identity ≥95%). Microbial diversity was well captured considering that rarefaction curves reached the plateau for all the analysed samples (Figure S2). The list and abundance of organisms identified in the samples, annotated irrespectively to the taxonomic ranks, and supported by at least two reads, are reported in Table S2. A total of 65 bacterial families and 160 genera were identified, in line with what was previously reported in another microbiota analysis in rabbits (Hu et al., 2021). When considering only families and genera supported by at least 2 reads, numbers decreased to 50 and 114, respectively (Table S3 and Table S4). The most abundant family was Clostridiaceae, followed by Moraxellaceae and Enterobacteriaceae (Figure 2). Clostridiaceae also appeared to be a common feature of all analysed samples (24%–57% of the 16S rRNA gene profiles) and genera (24%–63% of the 16S rRNA gene profiles) whereas the pattern distribution of the other bacterial families was not consistent across the investigated rabbits.

3.3 | Genomic regions associated with resistance/susceptibility to diarrhoea

In the first step, to maximize the phenotypic differences in the case and control association study, we considered only the severely affected suckling rabbits against the paired unaffected rabbits of the same litter (GWAS1). We then also run case and control genome-wide association analyses considering alternatively the suckling rabbits having the intermediate phenotype (i.e., mild diarrhoea) within the group of susceptible animals (GWAS2) or within the group of resistant animals (GWAS3).

Manhattan plots obtained by these three genome-wide association studies are shown in Figure 3a–c. In all three analyses, the genomic control inflation factor was close to one (Table 1), suggesting that the experimental design and adopted model properly corrected for population stratification. QQ plots of these analyses provided similar information (Figure S3). In the three genome-wide association studies, estimated genomic heritability was in the range of 0.19–0.21, with a standard error in the range of 0.09–0.10 (Table 1).

In GWAS1, we identified a total of six significant genomic regions located on five assembled rabbit chromosomes (OCU), including OCU2, OCU3, OCU7, OCU12, OCU13, and one genome scaffold (NW_003160554.1; Table 2 and Table S5). In this analysis, the most significant marker was on OCU3 ($P = 2.98 \times 10^{-07}$). Suggestively significant markers were identified in other eight chromosomes (Table S5). All the significant genomic regions were confirmed in GWAS3 (Table 2 and Table S5), where, for several markers, the level of significance increased, potentially suggesting that including rabbits with mild signs of diarrhoea in the group of resistant rabbits could better describe the genetic underlying components of resistance to diarrhoea in suckling rabbits. In GWAS3, the most significant marker (that also was the most significant across all three genome-wide association studies) was in the unassembled scaffold ($p = 9.50 \times 10^{-09}$). Some of the

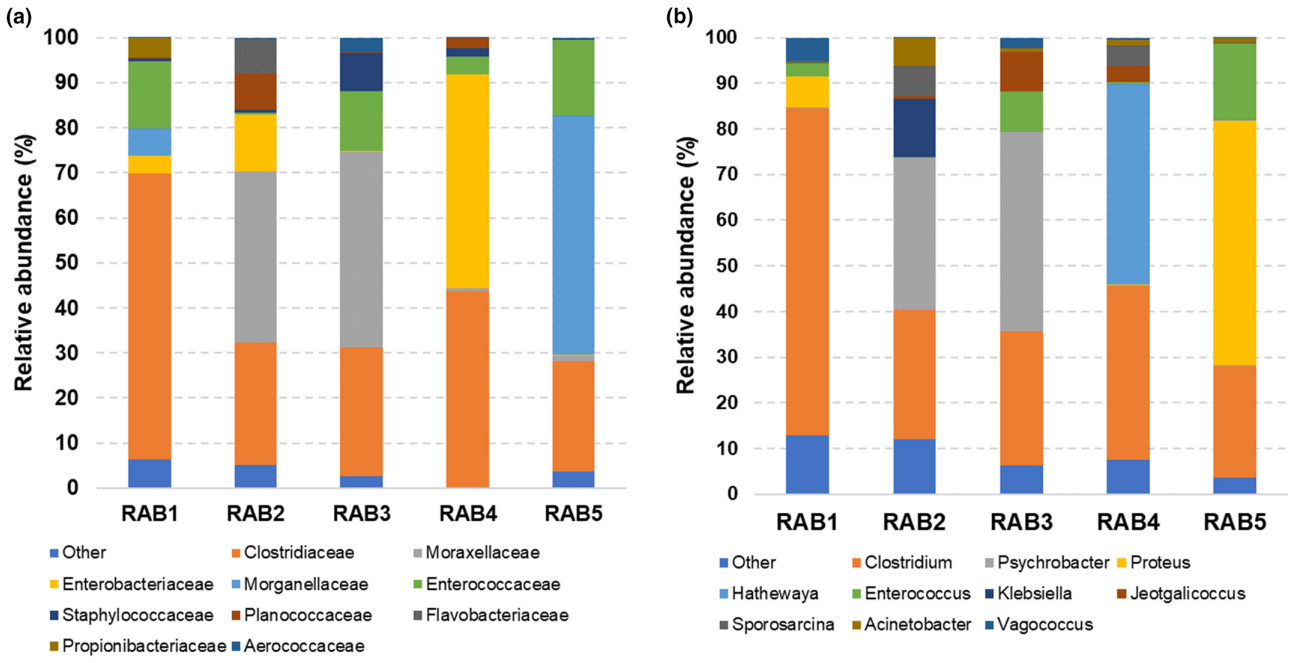

FIGURE 2 Bacterial 16S rRNA gene (V3 region) sequencing results of the content of rectum of five rabbits affected by severe diarrhoea. (a) Relative abundance plot of bacterial families; (b) relative abundance plot of bacterial genera. Plots show the ten most abundant taxa; the remaining taxa are grouped under the label "Other." The full list of families and genera are reported in Tables S3 and S4, respectively. [Colour figure can be viewed at wileyonlinelibrary.com]

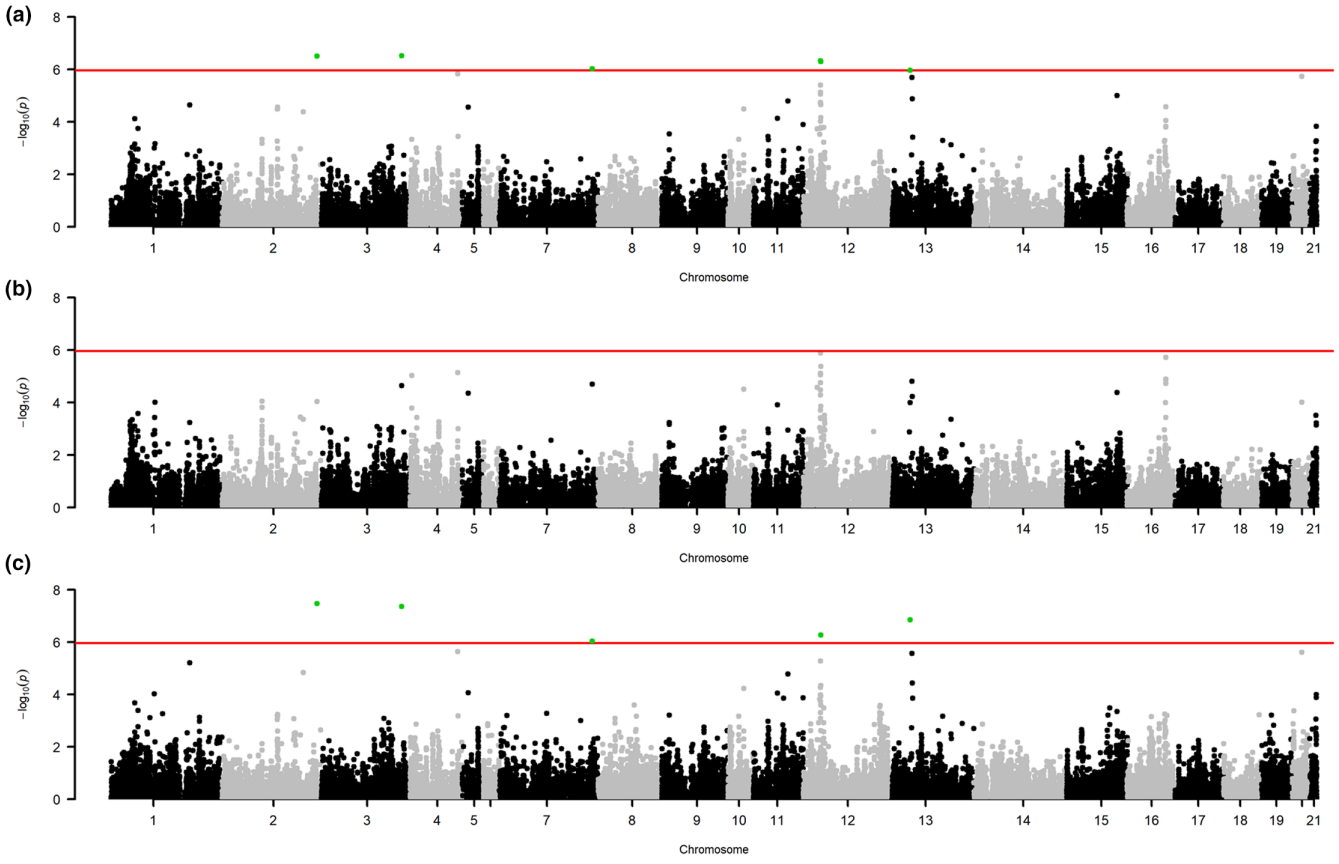

FIGURE 3 Manhattan plots of the genome-wide association studies (GWAS1-3) for diarrhoea in the suckling rabbits. (a) GWAS1: severely affected rabbits (susceptible) against the unaffected rabbits (resistant). (b) GWAS2: affected rabbits (with severe and mild symptoms of diarrhoea) against the unaffected rabbits (without any symptoms of diarrhoea). (c) GWAS3: severely affected rabbits against resistant (without any symptoms and with mild symptoms of diarrhoea) animals. Each dot represents a SNP. The red line identifies the significance threshold. [Colour figure can be viewed at wileyonlinelibrary.com]

suggestively significant regions identified in GWAS1 were also present in GWAS3 (OCU1, OCU2, OCU4, OCU11 and OCU20; Table S5). None of the significant regions that emerged in GWAS1 and GWAS3 were also significant in GWAS2, even if some regions remained just below the significance threshold, including another evident peak on OCU16, which could just be noted (even if far below the significance threshold), only in GWAS1 (Figure 3a–c; Table S5).

F_{ST} analyses carried out considering the same three groups of animals defined in the genome-wide association studies ($F_{ST}1$, $F_{ST}2$ and $F_{ST}3$) obtained similar results to those already reported for the corresponding genome-wide association studies (Figure 4a–c; Table S6). Table 2 includes information on the match of the results between the genome-wide association studies and the F_{ST} analyses. The highest F_{ST} value was 0.092, for a marker in the unassembled scaffold NW_003160554.1 ($F_{ST}3$), followed by a value of $F_{ST}=0.082$ for a marker on OCU13 ($F_{ST}3$), pointing out a low/medium genetic differentiation between the two extreme groups at all genomic positions. This F_{ST} peak was in the correspondence of the same region identified in GWAS1 and GWAS3 (Table 2 and Table S6). One region that clearly emerged in $F_{ST}2$, which however was just below the significance threshold in GWAS2, was on OCU16, as we already mentioned above.

Combining all these analyses and comparisons (i.e., GWAS1-3 and $F_{ST}1$-3), three main multi-marker peaks emerged on OCU12, OCU13 and OCU16. Results of the haploblock analyses of these regions are reported in Figure S4–S6. The top associated marker on OCU12 is included in the haploblock that encompasses the *dishevelled associated activator of morphogenesis 2* gene (*DAAM2*; Figure S4). This marker had a high LD ($r^2>0.88$) with the marker AX-147105744, located within the closest gene *molybdenum cofactor synthesis 1* (*MOCS1*). The roles of these genes thus far described in the literature and of those of the other close genes in this region (Table 2) do not point out any direct putative functional involvements in the described pathogenic condition.

The top associated SNP on OCU13 is located within the activating transcription factor 6 (*ATF6*, Figure S5) gene,

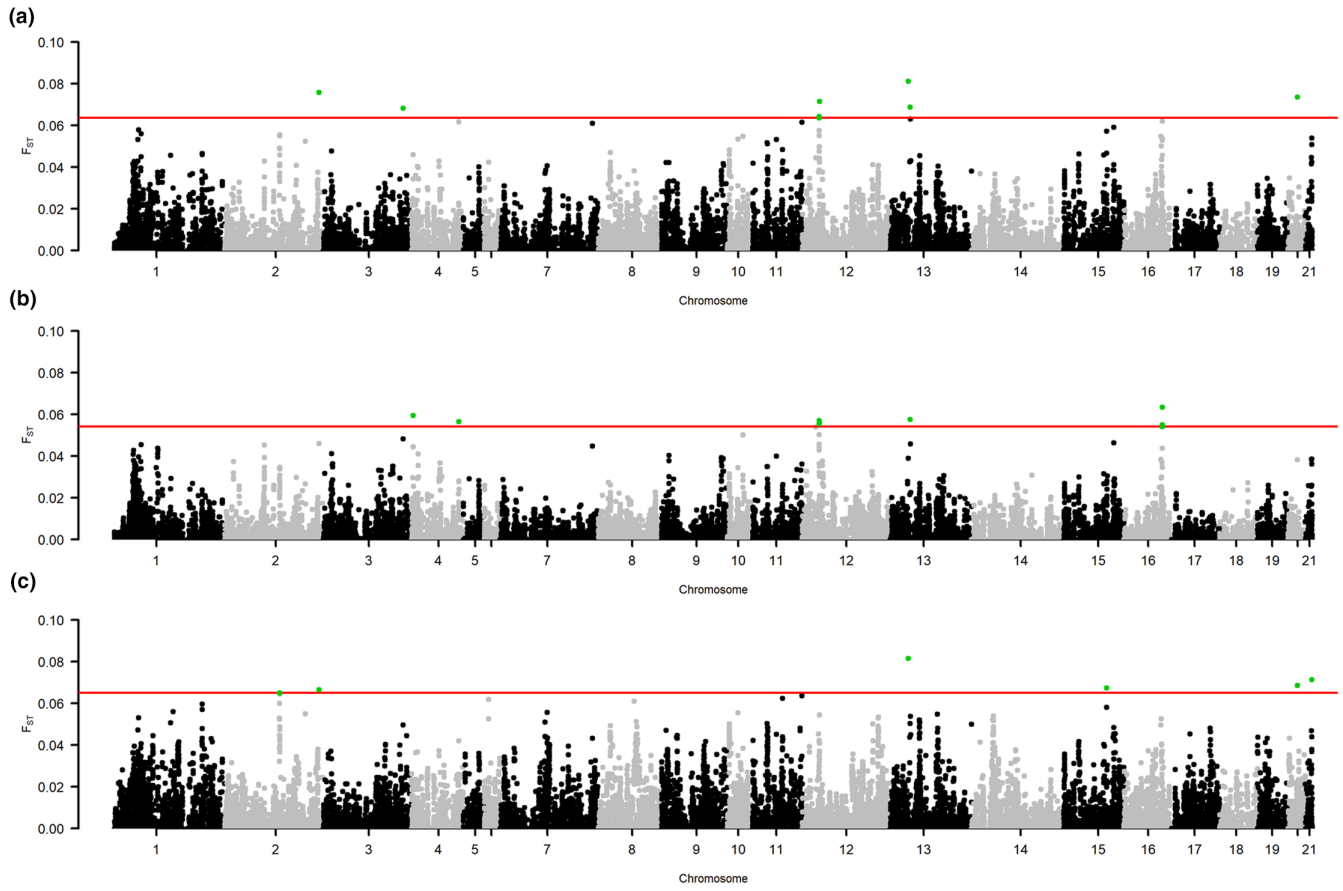

FIGURE 4 Manhattan plots of the F_{ST} analyses for diarrhoea in the suckling rabbits. (a) $F_{ST}1$: severely affected rabbits (susceptible, with severe symptoms of diarrhoea) against unaffected rabbits (resistant, without any symptoms of diarrhoea); (b) $F_{ST}2$: affected rabbits (with severe and mild symptoms of diarrhoea; susceptible) against the unaffected rabbits (resistant, without any symptoms of diarrhoea); (c) $F_{ST}3$: severely affected rabbits (susceptible, with severe symptoms of diarrhoea) against resistant (unaffected and with mild symptoms of diarrhoea) animals. Each dot represents a single nucleotide polymorphism. The red line identifies the F_{ST} threshold (99.98th percentile). [Colour figure can be viewed at wileyonlinelibrary.com]

associated in humans with leukocyte count. This region also encompasses several other genes that encodes members of a family of immunoglobulin Fc receptor genes found on the surface of many immune response cells and acting as adapter proteins involved in transmembrane signalling activity, mediating inflammatory signalling, and in the process of phagocytosis and clearing of immune complexes (*Fc epsilon receptor Ig*, *FCER1G*; *Fc receptor-like A*, *FCRLA*; *Fc receptor-like B*, *FCRLB*; *Fc gamma receptor IIa*, *FCGR2A*). The role of these genes might be very relevant in determining resistance/susceptibility to the investigated enteropathy in rabbits.

The top marker identified on OCU16 was part of a haploblock including the phosphatidylinositol-4-phosphate 3-pinase catalytic subunit type 2 beta (*PIK3C2B*) gene (Figure S6). PIK3C2B plays a role in signalling pathways involved in several mechanisms, including intracellular protein trafficking and translocation of proteins to membranes. The marker had moderate LD ($r^2 > 0.53$) with the marker AX-147156372, located close to the other groups of genes (*REN*; ENSOCUG00000033800 orthologous to *KISS1*; ENSOCUG00000028025, orthologous to *ETNK2*) whose known functional roles do not indicate obvious and direct involvement in the investigated disorder.

Functional enrichment analysis did not return any significant enriched terms or processes when using annotation information for genes included in all highlighted genomic regions. We also tested only those genes present in the three regions (OCU12, OCU13 and OCU16) where main peak markers were identified; also in this case, the queried databases and resources did not return any enriched term.

4 | DISCUSSION

Enteropathies, which mainly affect suckling and growing rabbits, are considered among the most important challenges that the rabbit meat industry is facing. These pathogenic conditions are responsible for high morbidity and mortality rates in affected rabbitries, causing problems on the sustainability of the rabbit farming industry (Solans et al., 2019). Selection and breeding to increase genetic resistance to enteropathies could be part of the solution of these problems. However, the collection of useful phenotypes for this aim encounters some practical difficulties. Simple clinical symptoms of enteropathies in commercial populations (e.g., diarrhoea, bloated abdomen or death of the animals) are the only phenotypes that can be routinely recorded, without identifying any clear pathogenic origin and without establishing all potential co-factors. Therefore, the complexity, heterogeneity and multifactorial causative agents determining enteropathies in rabbits and, in turn, the low heritability of the related traits, make very difficult to establish effective breeding programmes aimed to increase resistance to this pathogenic condition (Garreau et al., 2012, 2021; Gunia et al., 2015; Shrestha et al., 2020). Similar problems related to the records of useful phenotypes are also encountered in planning experimental designs where disease states could be associated with variability at the genome level with the aim to dissect the genetic factors determining resistance/susceptibility to enteropathies in meat rabbits.

In this study, we took advantage from the presence, in the same litters, of both suckling rabbits showing clear signs of severe diarrhoea and others without any signs of diarrhoea (or just mild diarrhoea) till weaning age. Within this suckling rabbit population, we nested case and control studies based on genotyped markers covering the whole rabbit genome. Suckling rabbits were therefore classified as resistant and susceptible to a severe enteropathy, using as proxy the external symptom of severe diarrhoea. This simple approach, in this paired design, however, tended to increase heritability of the investigated condition. Genomic heritability of resistance/susceptibility to severe diarrhoea estimated in this experimental design ($h^2_G = 0.19–0.21 \pm 0.10$) was higher than the heritability of enteropathies estimated in growing rabbits using pedigree-based information ($h^2 = 0.08 \pm 0.02$; Garreau et al., 2008). When we run other genome-wide association analyses with a linear mixed model that included all three classes of diarrhoea symptoms (unaffected, mildly affected and severely affected) taking into account all available fixed factors (litter, bucks, etc.), genomic heritability was almost zero (data not shown). These results suggest that the case and control study designed within litter was able to capture a quite relevant fraction of genetic variance that could not be preserved in any other models due to the complexity of the environmental factors outside litter that are also those that in most studies complicated the genetic analyses of resistance to enteropathies and other related diseases in rabbits.

The high incidence and mortality that we observed in our study is similar to what was reported in previous studies in other growing rabbit populations for similar enteropathies or related problems (Garreau et al., 2008; Solans et al., 2019), confirming that when rabbitries experience outbreaks of these types of disorders, the economic impact is usually very relevant. As next steps, we will further evaluate the incidence of enteropathies in the growing phase of the same commercial meat line used in this study, the heritability of this condition in the growing animals and the genetic correlation with other production traits.

The detailed characterization of the rabbit enteropathies is usually very complicated due to the potential

heterogeneity and co-presence of multiple causative factors, which would need detailed necroscopy, histological preparations and several analytical methods to detect and quantify multiple potential pathogens. These are the main reasons why thus far, despite several attempts, it has not been possible to clarify the biological components and agents of enteropathies and related disorders in rabbits. In the field conditions in which we nested our study, it was not possible to obtain specimens from all involved suckling rabbits (as the healthy animals could not be sampled); therefore, we opted for a first characterization of the diarrhoea microbiota of the deceased rabbits, sampled from different litters, that may correspond to the final stage of the disease. Despite the observed heterogeneity of the microbiota profiles across samples, also reported within experimental design in other studies (Chen et al., 2022; Wang, Fan, et al., 2022), the most consistent bacterial family and genus across all investigated specimens was Clostridiaceae (24%–57% of the reads of the 16S rRNA gene profiles) and *Clostridium* (24%–66% of the reads of the same profile), respectively, which might include pathogenic agents or might provide a peculiar signature of the resulted disease status. The genus *Clostridium* has been reported by the studies of Velasco-Galilea et al. (2018) and Hu et al. (2021) among the most abundant genera found in samples collected from the large intestine of healthy rabbits; therefore, it is possible that most of the species of this genus constitutes the normal cecum cellulose-degrading symbiotic microorganisms. It is however well known that some *Clostridium* species, including *C. spiroforme* and *C. piliforme*, can cause gastrointestinal disorders in rabbits (e.g., enteritis, enterotoxemia), with severe diarrhoea and high mortality (Oslesbee & Lord, 2020). It is also interesting to note that Bäuerl et al. (2014) reported a greater presence of this genus in caecal microbiota of adult rabbits affected by epizootic rabbit enteropathy (ERE) than in healthy animals. It could be possible that the pattern we observed in rabbit with severe diarrhoea could resemble a general situation of rabbit enteropathies, even if we did not have the possibility to verify the microbiota profiles of the healthy suckling rabbits and obtain a precise comparative analysis between cases and controls. Therefore, other studies will be needed to further explore the role of *Clostridium* species in determining the observed enteropathy in the suckling rabbits of the investigated meat line.

The applied genomic analyses, that were based on the within litter case and control design, were able to identify a few genomic regions associated with the resistance/susceptibility of the enteropathies that occurred in suckling rabbits, for which symptoms of diarrhoea were used as proxy. The results were obtained combining different genome-wide association studies and F_{ST} analyses. With these two genomic approaches, results derived from the same groups of rabbits (GWAS1-3 and F_{ST}1-3) were almost completely overlapping. This could be expected as genome-wide association studies and F_{ST} analyses in our experimental design rely on allele frequency differences between the contrasted groups of animals. Combining the three genome-wide association studies and the corresponding F_{ST} analyses, a total of eight genomic regions (on OCU2, OCU3, OCU7, two on OCU12, OCU13, OCU16 and on an unassembled scaffold) had significant SNPs and/or markers that trespassed the F_{ST} percentile distribution. Among these regions, three main peaks of SNPs were identified on three different chromosomes (OCU12, OCU13 and OCU16). The current available annotation of these genomic regions identified interesting candidate genes on OCU13: *FCER1G*, *FCRLA*, *FCRLB* and *FCGR2A* are involved in the biological mechanisms associated with the immune innate systems. These genes encode members of a family of immunoglobulin Fc receptors that are usually located on the surface of many immune response cells, in particular B cells. These receptors are involved in several different mechanisms, including antibody-dependent cell cytotoxicity, phagocytosis, allergic reactions, and transcytosis of immunoglobulins via their ability to bind immunoglobulin (Ig) constant regions (Ben Mkaddem et al., 2019; Bournazos et al., 2020). According to the role of these genes included in one of the major QTL we identified in this study, it is tempting to speculate that the resistance of some of the suckling rabbits and the susceptibility of others to an enteropathy form occurring in the pre-weaned period might be related to the possibility to transport IgG and enhance or not immunity derived from colostrum or through the fetal exchange at the placental level. This hypothesis could also justify the potential maternal effects on enteropathies, as demonstrated in other studies (García-Quirós et al., 2014). Further studies are needed to demonstrate the derived inferences that we based on the interesting results that pointed out on a putative relevant role of the genes included in the QTL located on OCU13.

From the obtained results, it is also clear that the genetic factors involved in the resistance to this enteropathy might be more complex, where more genes (most of which not well characterized and with few information on their roles) might be involved. Other two main QTL regions, on OCU12 and OCU16, emerged from this study and a few additional significant markers were also observed on other chromosomes. The most significant marker, however, was located in an unassembled scaffold, which, unfortunately, does not contain any annotated gene. The currently available *Oryctolagus cuniculus* reference genome (OryCun2.0) should be substantially improved, as it still contains many other unassembled portions and gaps that prevent, in some cases, the extraction of important genomic information

derived by genome-wide association or signature of selection studies (Ballan et al., 2022; Fontanesi et al., 2021). It is also interesting to note that none of the genes for which markers have been associated with non-specific digestive disorders in growing rabbits of a few Chinese lines (Chen, Zhang, et al., 2013; Fu et al., 2014, 2015; Li et al., 2018; Liu et al., 2013, 2017; Yang et al., 2013; Zhang et al., 2011; Zhang, Zhang, Chen, et al., 2013; Zhang, Zhang, Peng, et al., 2013) were included or were close to the genomic regions where we identified significant or suggestively significant SNPs. These results suggest (i) that different types of disorders were considered in these previous studies or (ii) that different rabbit lines might have different genetic backgrounds that would not provide the same genetic information in association studies or (iii) that the simple candidate gene approaches that was applied in the previous studies did not have the possibility to obtain replicable results in other contexts.

5 | CONCLUSIONS

Breeding to increase disease resistance is becoming fundamental to further support the development of a sustainable meat rabbit production system, where the incidence of the economic losses derived from many diseases is very relevant. At the same time, selection plans to pursue these objectives can reduce the use of antibiotics and improve animal welfare. The results obtained in this study demonstrated that resistance to an enteropathy occurring in suckling rabbits is in part genetically determined and can be dissected at the genomic level. The experimental design that we applied in field conditions based on a commercial population increased the probability to identify genomic regions associated with the resistance to diarrhoea, used as proxy of the effect of the investigated enteropathy. Based on this field-based experiment, this study provided for the first time a genome-wide association analysis that identified a few significant markers and genomic regions associated with the considered phenotype. We are working to implement the associated markers in the selection plans of the investigated meat rabbit line, increasing the frequency of the favourable alleles of the three main QTL regions. Additional studies are also needed to further refine and confirm the obtained results and evaluate the hypothesis of the potentially relevant role of members of a family of immunoglobulin Fc receptor genes on resistance to enteropathies in suckling rabbits.

AUTHOR CONTRIBUTION

L.F. designed the study, interpreted the results, and obtained funding. L.F. and S.B. wrote the paper. S.B. and G.S. conducted data and bioinformatic analyses. D.F. and A.F. provided samples and data. A.R., V.T. and F.B. contributed to data production and laboratory analyses. All authors read and approved the submitted version.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have any competing interests.

DATA AVAILABILITY STATEMENT

Genotyping data reported in this work can be shared after signature of an agreement on their use with University of Bologna. Sequencing data (16S rRNA) are available in the EMBL-EBI European Nucleotide Archive (ENA) repository under the study accession PRJEB64894.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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