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Mouse embryo rederivation and other assisted reproductive techniques and their impact on experimental results

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Summary

The use of assisted reproductive techniques in laboratory animal science, especially in rodents, has become a common procedure before the introduction of animals into animal facility SPF areas or as a tool to optimize animal use and fulfil the implementation of the 3R's. Some of these techniques, as it is the case of mouse embryo transfer, will have impact on the animal microbiota and, consequently, will be determinant for experimental results. Animal experiments are many times under scrutiny due to the lack of reproduction and, the mouse microbiota may, in part, be responsible for this issue. This work involved two main parts: optimization of ART's protocols with the aim of improving results and reducing animal use and a second part dedicated to the characterization of one animal model (the VCMSH2$^{LoxP/LoxP}$ mouse) in different areas of the facility to mimic two different microbiotas, before and after rederivation.

Experiments involving mouse embryo incubation led us to conclude that collecting embryos at a 1-cell stage (0.5 days post coitum (dpc)) after female superovulation and allowing the passage to a 2-cells stage (1.5 dpc) produced significantly higher number of embryos for the 3 backgrounds studied (C57BL/6J, B6*129 and FVB/N). Removing embryos earlier from the oviduct under the effect of high doses of PMSG and hCG seems to be beneficial for embryo development. Superovulation is known for its negative effects on embryo development and optimization of mouse embryo collection timings can have a very positive impact on the number of superovulated females.

Experiments to determine the effect of light cycles and timing of hormone administration in superovulation protocols revealed that 3-week-old females produce higher number of oocytes when hormones are given at 7 pm (the closest tested schedule to the beginning of the dark cycle) and 3 pm. However, when older females are used, even when optimal ages are used, at 4 and 5 weeks of age, better results were obtained at 7 pm. Both weight and estrous cycle phase were not considered to be significative variables in this model. At 3 weeks of age, female's puberty was not yet achieved and results obtained could be explained by the fact that, in older females, closer to the puberty onset, synchronizing the female LH surge with the timing of hormone administration produces better results.

Mouse embryo transfer was studied in C57BL/6J and B6*129 mouse embryos using different conditions. Unilateral transfers using wild type animals revealed that lower number of embryos are able to produce higher success rates when compared to high number of embryos. Bilateral transfer success rates seem to be related to strain: B6 embryos produce better results when higher number of embryos were transferred but B6*129 embryos had better results when lower number of embryos were used, which may be explained by the size of pups. This result emphasizes the idea that bilateral transfers should only be used

when a minimum number of embryos is available and that numbers as low as 6 embryos transferred unilaterally result in higher success rates when compared to larger number of embryos. Thus, these results can optimize the use of donor and recipient females.

The mouse model VCMSH2$^{LoxP/LoxP}$ was characterized in two distinct areas of the i3S animal facility to evaluate the GM impact on phenotypic characterization. Significant differences in the GM of these mice were found in alpha-diversity (Shannon index) measures, reflected in differences in the abundance of each microorganisms and shifts in the representativity of different phylum (Firmicutes and Bacteroidetes). Beta-diversity revealed differentiation of mice by the two zones. Tumour location had distinct patterns (although not statistically significant) in the two groups and lactoferrin levels were also different. Small differences in inflammatory levels were equally found. This experiment reflects the importance of GM characterization as a tool to reduce variability and improve experimental reproducibility.

Sumário

O uso de técnicas de reprodução assistida em ciências de animais de laboratório, particularmente em roedores, tornou-se um procedimento comum antes da entrada de animais em zonas livres de patógenos específicos ou como uma ferramenta para otimizar o uso de animais e concretizar a implementação dos 3Rs. Algumas técnicas, como é o caso da transferência de embriões, têm impacto na microbiota do animal e, consequentemente, serão determinantes para os resultados experimentais. Experiências com animais estão muitas vezes sob escrutínio devido à baixa reprodutibilidade e, a microbiota do murganho pode, em parte, ser responsável por este problema. Este trabalho envolveu duas partes principais: otimização de protocolos de reprodução assistida com o objetivo de melhorar resultados e reduzir o número de animais e uma segunda parte dedicada à caraterização de um modelo animal (murganho VCMSH2$^{LoxP/LoxP}$) em diferentes zonas do biotério para mimetizar duas microbiotas distintas, antes e após rederivação.

Experiências que envolveram a incubação de embriões de murganho permitiram-nos concluir que a colheita de embriões no estadio de 1 célula (0.5 dpc) após a superovulação de fêmeas e a passagem a 2 células (1.5 dpc) produziu um número de embriões significativamente maior nos 3 fundos genéticos estudados (C57BL/6J, B6*129 e FVB/N). A remoção de embriões mais cedo do oviduto, sob o efeito de elevadas doses de PMSG e hCG, parece beneficiar o desenvolvimento do embrião. A superovulação é associada a efeitos negativos no desenvolvimento do embrião e a otimização de tempos de colheita de embriões pode ter um impacto muito positivo no número de fêmeas superovuladas.

Experiências usadas para determinar o efeito do ciclo de luz e hora de administração de hormonas em protocolos de superovulação revelaram que fêmeas com 3 semanas de idade produzem significativamente mais oócitos quando as hormonas são administradas às 7 pm (o horário mais próximo do início do ciclo de escuro) e às 3 pm. Não obstante, quando são usadas fêmeas mais velhas, mesmo em idades ótimas, às 4 e 5 semanas de idade, os melhores resultados foram obtidos às 7 pm. O peso e o ciclo éstrico das fêmeas não foram considerados variáveis significativas neste modelo. Às 3 semanas de idade a puberdade não foi ainda atingida e os resultados obtidos podem ser explicados pelo facto de, em fêmeas mais velhas, a sincronização da administração das hormonas com o pico de LH produz melhores resultados.

Transferências de embriões foram estudadas em embriões de C57BL/6J e B6*129 usando diferentes condições. Transferências unilaterais com animais "*wild type*" revelaram que números de embriões mais baixos produzem taxas de sucesso mais elevadas quando comparado com números de embriões mais elevados. Taxas de sucesso de transferências de embriões bilaterais parecem depender da estirpe: embriões de B6 produzem melhores

resultados quando um número mais alto de embriões é transferido mas em B6*129, foram obtidos melhores resultados quando o número de embriões é mais baixo, o que pode ser explicado pelo tamanho das crias. Este resultado enfatiza a ideia de que transferências bilaterais devem apenas ser usadas com um número mínimo de embriões disponíveis e que números tão baixos quanto 6 embriões em transferências unilaterais resultam em taxas de sucesso maiores quando comparadas com transferências com números mais altos de embriões. Assim, estes resultados permitem otimizar o número de fêmeas dadoras e receptoras de embriões.

O modelo de murganho VCMSH2$^{LoxP/LoxP}$ foi caracterizado em duas zonas distintas do biotério do i3S para avaliar o impacto da microbiota na caracterização fenotípica deste animal. Foram encontradas diferenças significativas na diversidade alfa (índice de Shannon), refletidas em diferenças na abundância de cada microrganismo e alterações de diferentes filos (Firmicutes e Bacteroidetes). A diversidade beta revelou diferenças entre animais das duas zonas. A localização do tumor apresentou padrões distintos (apesar de não serem resultados com significado estatístico) e os níveis de lactoferrina foram também diferentes. Pequenas diferenças foram também encontradas nos níveis inflamatórios. Esta experiência reflete a importância da caracterização da microbiota intestinal como ferramenta para a redução da variabilidade e melhorar a reprodutibilidade experimental.

Abbreviations list

aa – amino acids
AMP's - antimicrobial peptides
APC - adenomatous polyposis coli
ARC – arcuate nucleus
ART - assisted reproductive techniques
ASF - altered Schaedler flora
ATP - adenosine triphosphate
COX-2 - cyclooxygenase 2
C-section – cesarean section
CRC - colorectal cancer
CTLA-4 - cytotoxic T-lymphocyte-associated protein 4
CTL's - cytotoxic T lymphocytes
DC's - dendritic cells
Dpc – days post coitum
DNMT - DNA methyltransferases
EGF - epidermal growth factor
ERα – Estrogen receptor alpha
ETBF - enterotoxic *Bacteroides fragilis*
FSH - follicle stimulation hormone
GF – germ-free
GIT - gastrointestinal tract
GM - gut microbiota
GnRH – gonadotropin releasing hormone
hCG – human chorionic gonadotropin
HPA – hypothalamus pituitary axis
IAS - inhibin antiserum
IBD – inflammatory bowel disease
ICB's - immune checkpoint blocker
ICSI - intracytoplasmatic sperm injection
IFN-γ – interferon gamma
Ig - immunoglobulin
IGF - insulin growth factor
IL - interleukin
iNKT – invariant natural killer T

IVF - in-vitro fertilization

KSOM - potassium supplemented optimized medium

LIF - leukemia inhibitory factor

LH - luteinizing hormone

LPS – lipopolysaccharide

MAMP's - microbe-associated molecular patterns

MLN - mesenteric lymph nodes

MMR – mismatching repair

NCOA-2 - nuclear receptor coactivator 2

ND - NADH dehydrogenase

NF-kB - nuclear factor kappa-light-chain-enhancer of activated B cells

NLR – NOD like receptor

NSAID - non-steroidal anti-inflammatory drug

NSET - non-surgical embryo transfer

PGF2α – prostaglandin F2 α

pks - polyketide synthase

PMSG – pregnant mare serum gonadotropin

PSP - pseudopregnancy

POA - pre-optic area

PD-1 - programmed cell death protein 1

TLR - Toll-like receptor

T reg's - regulatory T cells

Rag1 - recombination activating gene 1

Reg3 - regenerating islet-derived protein 3 gamma

ROS - reactive oxygen species

SCFA – short chain fatty acids

SCN - suprachiasmatic nucleus

SPF – specific pathogen free

STAT3 - signal transducer and activator of transcription 3

TGFα - transforming growth factor alpha

TGF-β – transforming growth factor beta

TNFα - Tumor necrosis factor alpha

Introduction

The use of laboratory animals for research purposes is a key tool for the development of biological sciences from basic to applied research. Animals still have an important role on the comprehension of basic physiological and disease mechanisms and despite all the alternative methods available we are still at a point where a complete organism cannot totally be replaced. The latest report (2015-2017) on animal use at the European Union refers that 9.3 millions of animals were used for scientific research, of which 62% were mice [1]. To this number, we also have to add the animals used for the maintenance of breeding colonies of wild type and genetically modified animals.

One of the advantages of using mice as a model is the ability to use genetically modified mice either from already available models or by producing the desired mutation. The introduction of the CrisprCas9 technology made the production of genetically modified mice easier and faster, making the access to genetically modified mice much facilitated and so the exchange of animals between animal facilities. Sharing different models contributes for scientific development but is challenged by concerns related to the introduction of animals into a different facility/environment, especially with the increased awareness of the microbiota impact on experimental results. To control the microbiota and to achieve some level of microbiological standardization, rederivation of mice through embryo transfer techniques is a commonly used method.

Assisted reproductive techniques (ART) are implemented in most of the animal facilities as a tool to prevent facilities contamination with undesired agents (embryo transfer – rederivation), to improve the reproductive performance (*in-vitro* fertilization (IVF), superovulation) and in some cases for colony management and genetic preservation (cryopreservation). The use of ART, especially embryo transfer, contributes for an easier and safer exchange of animals between facilities and can also overcome breeding difficulties.

The main techniques used as ART (superovulation, IVF and intracytoplasmatic sperm injection (ICSI)), all rely on the final step of embryo transfer, essential for the final outcome of having a litter. This process requires the use of a pseudopregnant female and, most of the time, this female has a distinct microbiological content when compared to the original mice to be transferred, resulting in changes in the microbiota which can, potentially, have an impact on experimental results and on the phenotypic characteristics of a specific strain. Several examples of this microbiota shift and the impact that it has on the phenotype are available in metabolic diseases, cancer models and neurological models. Hansen *et al* [2] highlighted different diseases that are shaped and influenced by the gut microbiota (GM), since diabetes to obesity, arthritis, inflammatory bowel disease, multiple sclerosis, colon

cancer and inflammatory or visceral pain, among others. The influence of the gut microbiota is not restricted to the adult animal. It's influence at the embryo level has also been demonstrated as short chain fatty acids (SCFA) produced by the mother GM are key for the differentiation of neural, intestinal and pancreatic embryonic tissues [3]. This is equally demonstrated by the use of germ free (GF) mice, whose offspring is characterized by a higher susceptibility to develop metabolic disease [3]. The GM potential to modulate different diseases is becoming more evident and so our awareness to consider it as a potential source of experimental variation and a determinant factor on experimental results. The application of the 3Rs, particularly the reduction of the number of animals used, should be a fundamental goal of those who need to use animals for scientific purposes.

The goal of this work was to further characterize and optimize different techniques commonly used in rodent animal facilities, focusing on superovulation, embryo culture and transfer and the impact embryo transfer may have on the gut microbiota of a mouse model of duodenum cancer ($VCMSH2^{LoxP/LoxP}$ mouse).

Assisted Reproductive Techniques: its limitations and impact on laboratory mice use

ART's are a common tool in laboratory animal facilities, as already stated. The introduction of new mice into a facility is commonly started by a rederivation process to eliminate possible unwanted microbiological agents and to standardize the microbiota. This is an essential point to harmonize animal models and to allow for a better reproducibility of results, a serious concern related to the use of animal models. The microbiota of the recipient female will serve as a basis for the offspring microbiota definition [4]. This controlled introduction of animals into a colony with a defined microbiological status is fundamental to prevent infectious diseases with a significant impact on animal welfare and on experimental outcomes. Infectious diseases have been shown to have a significant impact on experiments: detailed clinical outcomes of different infectious agents were described by Nicklas *et al* [5] and specific considerations for genetically modified animals were also stated by Franklin Craig [6]. Immunodeficient animals are particularly susceptible to clinical signs as a result of infection with pathogenic and opportunistic agents. IL10 KO mice, as an example, develop typhlocolitis when infected with *Helicobacter hepaticus* [6] and *Pasteurella pneumotropica* can lead to orbital abscesses in *Cd28* mice or pneumonia in CXCR2 mice [6].

Embryo transfer protocols are most of the times used together with other techniques, namely superovulation and embryo culture. Together, these techniques are key to reduce the number of animals used and to increase the efficiency of the process. Nevertheless,

none of the referred techniques is absent of side effects. The effects of different ART's on the cardiovascular system are well documented by the increase in oxidative stress in mesenteric resistance arteries produced via ART's [7], endothelial dysfunction [8] and other cardiometabolic effects in pups are also described extensively by Vrooman *et al* [9]. Other effects such as low birth weight, pre-term birth and birth defects have been associated with the use of ART's [10], as well as reduced growth and glucose intolerance [11]. Extensive work is also available on what is related to the epigenetic effects of superovulation, IVF and embryo culture. Examples include H19 gene expression dysregulation [12] or the loss of DNA methylation at imprinting control regions and disruption of imprinted genes expression [9]. These outcomes have a negative impact on the embryo and offspring development, but the advantages of ART's overcome these negative effects. Although it is possible to collect oocytes or embryos from a naturally synchronized female, the number of animals used for this purpose would be significantly higher. Techniques such as embryo microinjection for the generation of genetically modified mice would be challenging to perform due to the number of fertilized embryos that are necessary to ensure enough embryos for microinjection and a viable offspring. And without embryo transfer, the microbiological control of mice colonies would be certainly associated with a higher risk of microbiological agent's introduction, compromising also the microinjection of embryos and embryo manipulation needed for the generation of genetically modified mice.

Other techniques related to ART's, such as IVF and cryopreservation, that were not the aim of this work, are also essential tools for the maintenance of genetically modified and wild type strains. Although these techniques also display negative impacts on the embryo development, growth and susceptibility to different diseases, the potential of such techniques overcomes the disadvantages. IVF can facilitate the overcome of reproductive problems and is also essential if cryopreservation of sperm is used, which in turn is essential to colony management and genetic stability of mouse lines. Both of these rely on embryo transfer for a final successful outcome and are also associated with potential adverse impacts on the embryo and offspring. IVF seems to be related to actin cytoskeletal disorganization, leading to a deficient chorion and allantoid formation; placental nutrition is also impaired duo to alterations in hematopoiesis and vascularization and processing of amino acids and energy [13]. Genetic disruption and abnormal methylation and expression of imprinted genes are also related to IVF [13, 14], an effect that seems to be cumulative with other ART's, especially superovulation [15].

Superovulation

Mouse reproductive physiology

The female reproductive capacity and the oocyte production is dependent on a cascade of events that rely on functional structures and production of sexual hormones. One of the primordial organs responsible for this is the hypothalamus, formed at the fetal embryonic stage of E10.5, that starts producing mRNA encoding for the production of gonadotropin releasing hormone (GnRH) at E13.5 [16]. GnRH produced by the hypothalamus stimulates the anterior pituitary to produce follicle stimulation hormone (FSH) and luteinizing hormone (LH) which exert action on the ovaries. In turn, the production of ovarian steroids will play an important role through negative and positive feedback mechanisms on the hypothalamus-anterior pituitary axis to control the cyclic release of GnRH and gonadotropins production. FSH and LH are present at birth, although the reproductive system at this time is not completely mature and not functional [17] and receptors for FSH and LH aren't present until 3 to 5 days of age [18].

FSH and LH play an important role during follicle growth and differentiation, together with other molecules. A large number of primordial follicles are present at birth, but only a small proportion will reach the adult phase [17, 19], despite some authors also consider the hypothesis for new primordial follicle development through life [20]. The primordial follicles are involved in an initial recruitment process that starts before the onset of puberty [21] and involves intraovarian factors that initiate follicle growth. The follicle size only increases after day 12 to 15 of age [17]. As puberty onset arises, the cyclic recruitment starts due to the higher levels of FSH. As a consequence, primordial follicles start growing and thus the oocyte and the surrounding cells in a gonadotropins independent process [17, 22], but dependent on the presence of the inner oocyte and intraovarian factors. During this process there is a dominant follicle selection, that in the case of rodents, corresponds to several dominant follicles, whose production of FSH will exert a negative feedback action on the hypothalamus-pituitary axis controlling the growth of other smaller follicles [21]. This controlling mechanism also seems to be related to endogenous factors produced by the follicles, by the FSH levels [23] and other molecules such as insulin growth factor (IGF) [21]. The development of this primordial follicle continues with the differentiation of the surrounding granulosa cells giving origin to a primary follicle and later, when both thecal and granulosa cells are differentiated, to a secondary follicle. Pre-antral and antral follicles development is again dependent on the levels of FSH and although LH and hCG are important for their growth too, FSH has the most determinant role [21]. The final stage for follicular development is the antral follicle or Graafian follicle, whose larger size is now

dependent on the increased volume in the antral cavity as mitosis are less frequent. This final stage is very dependent on FSH levels, as already stated, but other factors are also important for the final steps that will lead to ovulation: IGF-1, epidermal growth factors, fibroblast growth factor-2, interleukin-1 [21] and growth hormone [21, 24]. The consistent increase in FSH will cause a prolonged period with high levels of estrogens and consequently, an LH surge. Twelve hours later the follicle rupture occurs, followed by ovulation and luteinization of granulosa cells [17, 25].

Mice are spontaneous ovulators and the circadian rhythm seems to determine the LH surge and the ovulation time by controlling neuroendocrine responses at the suprachiasmatic nucleus (SCN) [17, 26]. The circadian rhythm also seems to be associated with other areas besides the SCN, contradicting the classic idea of ovulation timing control: the ovary itself controls the timing of ovulation through the production of prostaglandin E2 and prostaglandin F2α, whose inflammatory action is important for the follicular rupture [26]. Prostanoids action is dependent on the activity of cyclooxygenase 2 (COX-2) under the control of CLOCK BMAL1 [26], which are key genes related to the rhythmicity control and fertility. The circadian rhythm control is a complex issue where other molecules participate such as vasopressin, transforming growth factor (TGFα) and prokineticin-2 [27].

One of the most important mechanisms related to the circadian rhythm is melatonin produced by the pineal gland, whose action regulates not only reproductive behavior but also sleep, cancer and diabetes. Melatonin is related to seasonal breeding, allowing a precise control of the ideal time for breeding, matching the best environmental conditions and offspring survival [28]. Melatonin levels are higher during the night period, and lower during the day and the number of hours of light animals are exposed to, will determine the levels of melatonin and consequently, the production of GnRH [29, 30].

Another key molecule for reproduction is kisspeptin, which has been described as a potent GnRH stimulator and determinant for the onset of puberty [31-34]. Kisspeptin is related to 3 main roles: it drives the onset of puberty; in adults it is related to the tonic episodic secretion of GnRH/LH controlled by the negative feedback of gonadal hormones and is also responsible for the pre-ovulatory LH surge [34]. The pre-ovulatory surge and the onset of puberty are affected by kisspeptin although, downregulation of kisspeptin does not completely blocks these phenomena's in young animals; in adults however, it will cause disruption of the estrus cycle suggesting the existence of compensatory mechanisms at early phases of development [34]. The number of kisspeptin expressing neurons is higher in females when compared to males and it is also known that, these neurons act through GnRH expressing neurons to trigger olfactory cues responsible for the female preference for different males and sexual motivation in females [33].

The onset of puberty is an important landmark for reproductive ability. In females, it is characterized by vaginal opening, cornified vaginal smears and first estrus, mating behavior and reproductive ability to develop and maintain a full pregnancy [17]. The onset of cyclicity is perhaps the more accurate landmark for the puberty definition but the genetic traits responsible for the cyclicity onset and those responsible for vaginal opening and first estrus timing are distinct [35]. Several factors affect the onset of puberty. Photoperiod and day length affect the puberty onset with short day lengths delaying it [17, 36], through changes in kisspeptin release. The diet fat content and body fat can also affect puberty onset and high body fat has been related to early onset of puberty [37]. Leptin is a potential stimulator of the HPA as low levels of leptin were described as a puberty delaying factor [17], however, recent work has shown that the body fat is the main factor and not leptin [37]. Early stress life, such as low bedding levels, is also related to delayed vaginal opening time without affecting the estrous cycle [38]. Substance P accelerates puberty onset, demonstrated by the increased expression of Tac1 and Tacr1 before puberty [39]. Pheromone's role is also crucial for the timing of puberty onset: females exposed to male pheromones have an early puberty onset and exposure to female pheromones delays it [17, 40]. The mentioned factors are important for puberty onset but the main controller, as described earlier, is kisspeptin. Increased levels of kisspeptin before puberty as a result of estrogen stimulation is one of the main mechanisms for puberty onset [34] and selective ablation of estrogen receptors (ERα) in kisspeptin neurons results in an advance of puberty onset [32]. Vaginal opening is an important landmark related to puberty. Several authors describe the timing of vaginal opening in mice to be around 24 to 30 days of age [17, 41], a Bcl2 related apoptosis event [42]. Unlike other species, vaginal opening is not coincidental in mice with the first estrous cycle and it takes a mean number of 7 days for the first estrous cycle to occur after vaginal opening is detected [43].

Mice are spontaneous ovulators and the estrous cycle is relatively short, being completed at the end of 4 to 5 days [41, 44-47]. The cycle can be divided in 4 important phases: proestrus, estrus, metaestrus and diestrus, represented in figure 1. The main method for their classification is through a vaginal cytology, which should be performed daily during at least 14 consecutive days to determine its duration [45]. Proestrus is characterized by high levels of estrogen and by the end of this phase, the LH surge occurs; progesterone also starts to increase during the proestrus and the cytology is characterized by clusters of small rounded nucleated cells and no neutrophils are present at this stage [41, 44-47]; it is completed in less than 24h. At the end of the proestrus and beginning of the estrus, ovulation occurs, and females are receptive during this phase to a male. Estrus is, in this way, characterized by ovulation and corpus luteus formation; if pregnancy is not the final outcome, estrogens and progesterone start to decrease; cytological evaluation will show

anucleated epithelial cells and, some nucleated cells may also be present; it takes about 12 to 48h to be completed [41, 44-46]. Metestrus is characterized by low estrogen and progesterone levels; during the first half of metestrus, some neutrophils can be identified between the anucleated cells forming clusters but, by the beginning of the second half, the cytology consists mainly of neutrophils; this phase is completed in less than 24h [41, 44-46]. Diestrus is characterized by rising concentrations of estrogens, particularly by the end of the diestrus; it is characterized by less cells available for cytological evaluation with a predominance of neutrophils but very rare anucleated cells, with an increase in nucleated epithelial cells by the end of the diestrus; it can last for 48 to 72h [41, 44-46]. The different phases can also be determined in a less accurate way by visual observation of the external genitalia [46]. Cycles are continuous and dynamic, and anestrus is not a physiological state in laboratory mice. The cyclicity can be affected by the nutritional status, diseases, among other factors, and alterations to the light cycle can also cause irregular estrous cycles [48]. Prolonged exposition to day light induces longer estrous cycle in mice and grouping several females in the same cage exposed to a normal light cycle has a similar effect [49]. Male pheromones exposition has the opposite effect, shortening the estrous cycle [49, 50]. When females are kept in light cycles with longer dark periods, a larger variation can be found in the timing of ovulation, however, the timing of ovulation seems to be more dependent on the midpoint of the dark cycle than on the onset of ovulation [51]. Females with 4 days cycles and 5 days cycles seem to have a similar period for LH surge [50]. One study also refers the LH amplitude to be dependent on the intensity of the light but this effect may just be a normal response to an increase in the light intensity as wild rodents aren't often exposed to light [52].

Variation on the estrous cycle can be related to the strain. Some strains have more regular cycles, such as the C57BL/6J mouse and others have irregular ones (C3H/HeJ and DBA2/J) as described by Nelson *et al* in 1992 [53]. This is one of the reasons why prior characterization of the estrous cycle of a group of animals for at least 14 days is essential to determine the different phases in each strain/animal.

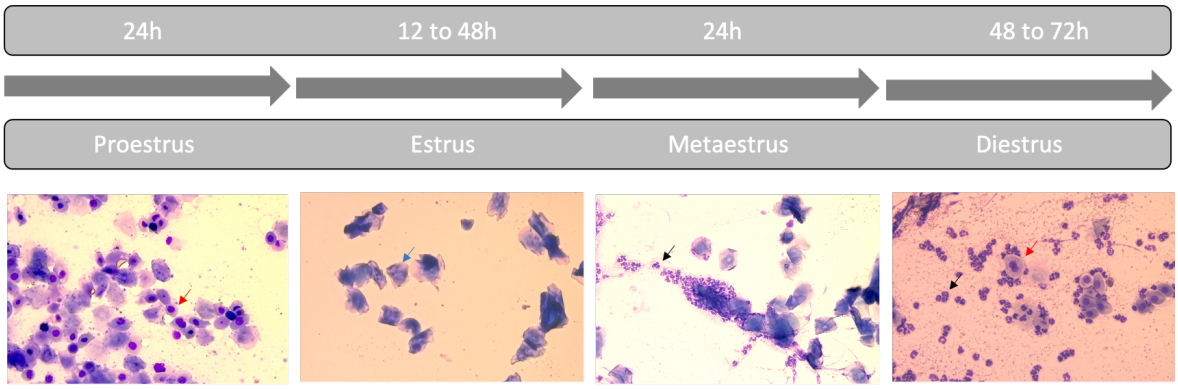

Figure 1. Estrous cycle phases, duration and vaginal cytology. Nucleated cells (red arrow); anucleated cells (blue arrow); neutrophils (black arrows)

Superovulation protocols

Mouse superovulation is a common procedure used to obtain large number of oocytes or embryos. It is being used for many years as a tool to synchronize females in the same phase of the estrous cycle, promoting follicle recruitment and ovulation. This protocol accounts for a significant reduction on the number of females needed as oocyte donors. Several researchers attempted during the last decades to optimize the protocol aiming for a reduction on the number of oocyte donors and an increase in the number of oocytes obtained. The most commonly used protocol consists in the injection of two hormones: pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), which mimic the effects of FSH and LH, respectively. The protocol requires the injection of PMSG, intraperitoneally, followed by hCG, using the same route, with an interval of 46 to 48 hours and an hormone dose of 5 IU for both PMSG and hCG [54]. The hormone doses have been explored by several authors throughout the years. Fowler, in 1957, described the use of 1 IU of PMSG followed by 2 IU of hCG with an interval of 40h or 3 IU of PMSG and the same dose of hCG as an efficient method for superovulation [55] and later, Christenson described PMSG and hCG doses between 2 and 16 IU to produce better oocyte yield, but doses higher than 24 IU were described as having a reductive effect on the oocyte number [56]. Doses of 15 to 30 IU were also described later on, with a plateau of 50 IU as the limit for oocyte increased production after PMSG/hCG stimulation [57]. The adverse effects of higher doses have however demand for an optimization at lower doses and most commonly, doses remain between 2.5 and 7.5 IU [54, 58-60]. From the different doses tested, 5 IU of PMSG followed by 5 IU of hCG is described as being a protocol efficient for different strains [60]. Variations to this protocol include the administration of two doses of PMSG, a week apart, before the administration of hCG, all at 5 IU, producing good results in C57BL/6J mice [60]. The administration of inhibin antiserum (IAS) has also been

described as an alternative to the classic protocol of PMSG/hCG. A combination of two progesterone doses (for estrous cycle synchronization at 2 mg/animal) followed by two doses of IAS and hCG (5IU) produced better results in C57BL/6J, BALB/cA, ICR and B6D2F1 mice when compared to PMSG/hCG administration [61]. A protocol consisting in the administration of IAS combined with PMSG (3.75 IU) followed by hCG (7.5 IU) injection 48 hours later also resulted in a higher oocyte yield when compared to the administration of IAS or PMSG followed by hCG [62].

Superovulation and factors affecting its efficiency

Several factors affect the outcome of superovulation, namely the oocyte yield and the quality of the oocytes. The most important factors that have been addressed in the past are female's age [60, 63-65], weight [60], hormone doses [55, 56, 60], interval between hormones [66], light cycle [67], strain [59-61, 64] and estrous cycle [58], as summarized in figure 2.

Superovulation is more efficient in younger females, duo to the fact that external hormones are administered in the organism at a time when internal gonadotropins are still at lower levels, before puberty (less than 32 days of age) [63], although some strains seem to respond better at 6 weeks of age such as the case of CD-1 mice [60]. These outcomes are also related to lower weights, as C57BL/6J, FVB/N, B6D2F1 and BALB/c mice respond better to superovulation at lower weights. In the case of B6 mice, groups of females between 5.5 and 16.2 g, corresponding roughly to 3 to 4 weeks of age, produce higher yields of oocytes [60]. Some initial works also described better outcomes for superovulation when females used were 25 days of age for C57BL/6N, DBA/2N, CD-1 and B6D2F1 strains when compared to females at 50 and 90 days of age [64]. CD-1 mice seem to have a contradictory behavior in the response to superovulation hormones but, this strain is also characterized by vaginal opening later (at 29 to 35 days) [68, 69]. Moreover, as described for C3H mice the interval between vaginal opening and starting cyclicity (the more reliable outcome of puberty onset) is not a fixed interval (C3H show vaginal opening before B6 and DBA mice but are the last to start cycling when compared to these two strains) [35]. Other internal mechanisms seem to impact the onset of puberty and the response of different strains to superovulation.

Apart from the efficiency measured as the number of oocytes produced after superovulation in pre-pubertal females, late reports state differences in the quality of blastocysts produced after superovulation of females at different ages: superovulated pre-pubertal females produce blastocysts with reduced microvilli and increased smooth surface area [70].

Little work is available on what is concerned to the light cycle and its impact on superovulation. Legge reported in 1994 the light cycle impact on superovulation stating that the administration of hormones 1 hour before the midpoint of the light cycle produced higher number of oocytes [67]. This work was performed using mature females from a hybrid strain (BALB/cBy x C57BL/6By), which are not ideal for superovulation purposes as younger females produce better oocyte yields. Other authors recommend the administration of hCG to be a few hours before the internal LH surge, which occurs 6 hours before ovulation or 15-20 hours after the midpoint of the dark cycle [71]. Another author described the LH surge to occur between 2 and 5 pm and ovulation at the beginning of the morning (lights on between 5 am and 7 pm) [72] To date, no information regarding the light cycle impact on superovulation of younger females is available.

Differences in superovulation are also related to the mouse strain used [59, 60]. These differences are reflected on oocyte's number and also on the response to different protocols. C57BL/6J produce higher oocyte yields (30.4 ± 2.1) when superovulated using two doses of 5 IU of PMSG followed by 5IU of hCG and the mean number of oocytes produced by this strain is higher than the number of oocytes produced by FVB mice using a protocol 5 IU of PMSG followed by 5 IU of hCG (15.1 ± 1.3), but lower than the mean number of oocytes produced by B6D2F1 using the last referred combination (45.3 ± 3.9) [60]. These variations show that efficiency of superovulation is affected by strain and that different superovulation protocols also produce different outcomes depending on the strain used as oocyte donor.

The interval between hormones was described as also being determinant for the superovulation outcome: Wilson *et al*. [66] described an interval of 30 to 50 hours to be related to a higher number of oocytes and, more than 60 hours reduces the number of oocytes released by the female due to atresia of some of these. The same author also pointed the importance of the timing between hCG administration and oocyte collection (16 hours ideally for higher oocyte yield) [66].

The estrous cycle has been described as another factor with potential to change the outcome of superovulation and contradictory results were achieved by different researchers. One study refers that, for CD-1 mice, the number of oocytes collected from animals in different phases of the estrous cycle was similar between groups, concluding that the estrous cycle phase does not have an impact on the number of oocytes collected after superovulation [73]. The results described in the referred work may however be interpreted with caution as oocyte quality and the estrous cycle at the time of superovulation seem to be related: oocytes collected from females at metaestrus were described as having a higher incidence of cumulus free oocytes, absence of polar bodies and intracytoplasmic mitochondrial aggregates [74]. Another author described lower pregnancy rates when

superovulation was performed during metaestrus and diestrus, using also CD-1 mice [75]. This last study evaluated pregnancy rates and not the total amount of oocytes released by a female and considered that high levels of Muc-1 at E3.5 females in metaestrus or diestrus at the time of superovulation could explain the lower pregnancy rates in these phases of the estrous cycle [75].

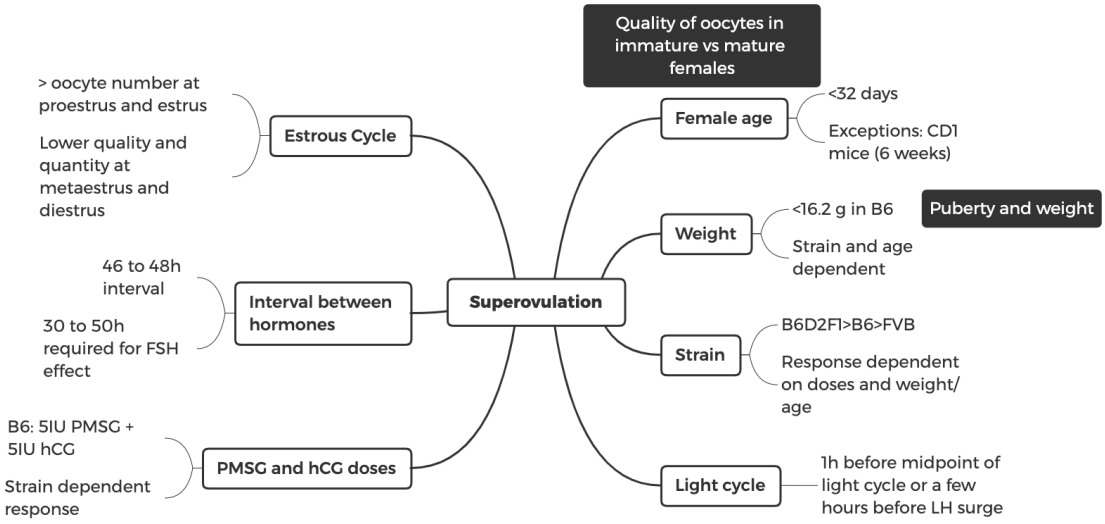

Figure 2. Main factors with impact on superovulation

Negative impact of superovulation on oocyte quality, embryo development and post-partum offspring health

The negative effects of superovulation are reflected at different levels and stages of embryo development and persist during animal development in the post-partum period. It is well known that superovulation is directly related to DNA alterations, especially on what is related to genomic imprinting through DNA methylation process, a key process for gene regulation and normal development. Superovulation, especially when high doses of hormones are used can change the activity of DNA methyltransferases (DNMT) [76] and a decrease in CpG methylation levels has also been reported in oocytes and blastocysts resulting from superovulated females [77]. Genes like *Mest* are affected by superovulation [78] and repeated superovulations are also associated with changes in DNA methylation levels and several genes expressed by the cumulus cells, such as *COX1*, *CYTB*, NADH dehydrogenase (*ND2* and *ND4)* [79]. While DNA methylation levels are decreased for genes such as *Snrpn*, *Peg3* and *Kcnq1ot1*, other genes have an increase in DNA methylation levels as a superovulation process effect, as it is the case of H19 [12, 80]. DNA methylation levels in the inner cell mass also increase with superovulation [81]. These

changes in methylation levels have an impact on embryo development and disease incidence in the offspring. On the other hand, sperm methylation patterns can be changed for at least 2 generations in the offspring of superovulated females [82].

Another outcome of superovulation is related to placental development, including placental overgrowth [15] and increased density of placental microvasculature in superovulated pregnant females [83]. The dysregulation of interleukin (IL) 33 also seems to be the cause of pregnancy disorders in superovulated females [84].

Embryo development is equally affected by superovulation and abnormal blastocysts, increased uterine reabsorption, reduced implantation sites and decreased fetal growth have been reported after female's superovulation [85, 86]. Embryos flushed right after ovulation and transferred into a pseudopregnant female also tend to develop better when compared to embryos submitted to culture or IVF [87]. Superovulated female's oocytes are related to a decreased quality with impaired maturation and functional ultrastructure of mitochondria [88].

A higher incidence of premature birth and low birth weight [11, 83, 89] are common in superovulated animals, probably related to alterations in lipid metabolism that affect both mother and fetus [90].

Many of these negative outcomes are particularly enhanced by the cumulative effect of several superovulation protocols, which can decrease fertility rates and embryo development in CD-1 mice [91], or the use of superovulation and other ART's in combination, such as incubation and superovulation resulting in higher impact on imprinted methylation patterns [92].

Mouse embryo culture

In-vitro maturation of preimplantation mouse embryos

One of the purposes for the use of superovulation is the collection of oocytes, one-cell embryos or eventually two-cell embryos for the purpose of microinjection, cryopreservation or embryo transfer for rederivation purposes. All of these applications require the collection of oocytes or embryos in a proper manipulation medium and in some cases a proper incubation medium [71].

For microinjection purposes, after superovulation, females are euthanized by cervical dislocation, the oviduct is isolated, and the cumulus cells removed by tearing the oviduct to collect 1-cell embryos that are then microinjected [93]. Two possibilities are then available for these embryos: to be kept inside an incubator up to a two-cell or blastocyst stage before

embryo transfer in the following day (two-cells) or days (blastocyst) or to be transferred at a one-cell stage to a pseudopregnant female on the same day [71].

Rederivation will also require female's euthanasia followed by cumulus cell isolation to obtain one-cell embryos [94] or a second method can alternatively be used by collecting the embryos at a two-cells stage, on the day after the mating (E1.5) by flushing the oviduct [95, 96]. Collecting the embryos at a one-cell stage and using overnight incubation has some practical advantages as workflow organization can be divided by two working days and in some cases, without depending of a second person to perform the transfers. On the other hand, skipping the overnight incubation speeds up the process and reduces the amount of time the embryos are kept outside of the uterine environment but, it requires usually a second person to perform the embryo transfer. Performing the embryo transfer at a two-cells stage has also the advantage that only fertilized embryos are used during the process and possibly allows for a more precise introduction of viable embryos into the oviduct of the recipient female.

Cryopreservation of mouse embryos is more commonly performed using zygotes [97], 2-cell embryos or morula [98-100]. In case embryos are cryopreserved in a 2-cells stage or morula, collection can also be done at the corresponding stage of development or, similarly to the embryo rederivation/embryo transfer process, development using culture can be performed after collection at a 1-cell/zygote stage.

Culture conditions, including medium, temperature, gas conditions will determine embryo ability to survive and also the quality of the embryos when using incubation. As already discussed, several work exploring the effect of superovulation on embryo development and implantation sites reduction was published however, the connection between superovulation and the timing of embryo collection to date, was not yet explored. Superovulation decreases the number of implantation sites [86] and for applications such as cryopreservation, embryo microinjection and embryo transfer for rederivation purposes, optimizing the timing of collection avoiding a reduction due to the superovulation effect on the uterine milieu is an opportunity for female donor reduction.

Embryo medium and its components

Using overnight culture is a common step during embryo manipulation and several protocols and medium are available including the most commonly used M16, KSOM, but also CZB and MTF [71]. For handling outside the incubator, M2 is the most appropriated [71].

Medium composition is key for a normal embryo development. During its initial development, embryos use lactate, aspartate and pyruvate as its main sources of energy, up to the 8-cell stage and are not able to use glucose, whose dependence occurs after

compaction [101]. The initial development of the embryo is possible in the absence of pyruvate if lactate and aspartate are present [102]. After the 8-cell stage, the embryo starts using glucose but aerobic glycolysis via lactate is still an important source of energy at this stage [101]. Glucose has been related to impairment of embryo initial development but, its negative effect seems to be related to high concentration of phosphate in the medium and removing it completely from embryo culture medium does not seem physiological as it is present in uterine fluids [71].

Apart from the components responsible for energy source, amino acids (aa) are also very important for the normal embryo development. Embryos have the ability to uptake aa and the incorporation of aa in the culture medium produces higher developmental rates as well as a normal mRNA production for several genes [71]. The supplementation of aa to KSOM produced normal levels of actin, glyceraldehyde-3-phosphate, dehydrogenase, IGF-I and II, among other elements [103]. In fact, the supplementation of KSOM with aa results in the development of zygotes to the blastocyst stage, with maintenance of normal structure and inner cell mass [104] as well as allowed the overcome of the 2-cell block in CF1xB6D2F1 mice [105]. The two-cell block occurs in embryos of some inbred and outbred strains developed *in-vitro* due to imbalances in the culture medium in a phase that the embryonic genome takes the control over the maternal genome [106]. It depends mainly on the oocyte strain, and not on the sperm strain origin [71] and adjusts in the concentration of KCl, NaCl, KH_2PO_4, glucose and pyruvate [106] proportionated conditions to overcome this blockage.

Other reported components in culture medium with benefits in embryo development include IGF-I and II [107, 108]. The incorporation of transforming growth factor alpha (TGF-α) and epidermal growth factor (EGF) improved embryo development [109], as well as L-glutamine [110], urokinase plasminogen activator [108] and antioxidants [111].

KSOM has been described as having better results when compared to other medium, such as the M16 and CZB but this outcome is strain dependent. When compared to Whitten's medium, one of the first used for embryo culture, KSOM affected the expression of significantly less genes [112]; higher apoptotic index was observed in embryos cultured in M16, from CBA and MF1 strains compared to embryos cultured in KSOM [113].

Incubation temperature, embryo density and volume as success factors

The success of mouse embryo culture is dependent on several factors, the most important embryo density, medium volume, temperature and gas atmosphere.

Recent work shows that mouse embryos develop better at 37ºC and that lower temperatures slow down embryo development but a slight increase to 37.5ºC accelerates it [114]. Experiments using B6D2F1 and B6SJLF1 mouse embryos revealed that pronuclear

embryos were more sensitive to temperature variations when compared to more advanced embryos [115]. These temperatures are important to be kept inside the incubator and minimized by avoiding manipulating the embryos for long periods outside the incubator.
Maintaining medium equilibrium is fundamental for embryo development. The introduction of sodium bicarbonate was essential to prevent pH unbalances outside the incubator. The gas content inside the incubator must also be controlled; exposition to O_2 should be minimized (5-7%) [116] and increases of O_2 of up to 20% impair embryo development [117]. Embryo density and medium volume are determinant for a successful embryo culture. It is known that individual culture of mouse embryos is not beneficial for the embryo development [71, 107, 117-119]. Using low numbers of embryos or single culture of embryos is related to slower cleavage rates and low number of embryos developing into a blastocyst stage as well as increased apoptosis [118]. The singly culture of embryos in 25 µL has poorer outcomes when compared to the culture of embryos in the same medium (KSOM + aa), in groups of 30 and using the same volume [119]; groups of 5 to 10 embryos in 25 µL also produce better outcomes when compared to single culture [109]; a concentration of 1 embryo/ µL, except when corresponding to single culture, was equally described as optimal for embryo development [107]. These better results in embryo development and passage to blastocyst seem to be related to the fact that when embryos are cultured in larger groups are exposed to autocrine and paracrine factors released by the embryos which are important for their development [71, 118].

Mouse embryo culture and embryo quality

Just like superovulation, mouse embryo culture also affects the quality of embryos. Several alterations were described from morphological, developmental and epigenetic alterations. Blastocysts resulting from *in-vitro* culture in KSOM have been characterized as having reduced inner cell mass as well as trophectoderm cells [120, 121]. The effects of embryo culture are reflected on the genetic content of these embryos. Metabolic, proliferative, apoptosis related and morphogenetic pathways are among the most commonly genetic pathways affected by embryo culture [120]. Effects on imprinted genes methylation with alterations of the *H19* [12, 15], *Grb10* and *Grb7* [122] were described for embryos resulting from embryo culture. Alterations in retrotransposable elements were also detected as a consequence of this process [120]. Moreover, animals resulting from *in-vitro* culture have increased systolic blood pressure, alterations in some metabolic and cardiovascular enzymes such as at the hepatic phospho*enol*pyruvate and serum angiotensin converting enzymes [121].

These effects are, according to some authors, less pronounced than the ones related to superovulation [89] but a cumulative effect between the two techniques and also with IVF has been described [15, 92].

Mouse embryo transfer

Embryo transfer is the final step that determines the success of several ART's from embryo manipulation to cryopreservation or mouse rederivation. Obtaining a live offspring is the ultimate goal of these techniques and all the efforts are, at this point, resumed to the female ability to maintain a full pregnancy and the litter capacity for survival after birth.
A delicate balance and communication between the embryo and the uterine horn is needed for a successful pregnancy to occur.
The outcome of embryo transfer is dependent on several factors but still, a high variation exists on how this technique can be implemented. The main factors are related to the recipient female, the surgical technique and the number and strain of embryos transferred [71, 95, 96], among others, which will be detailed in the next topics.
Using embryo transfer as a tool for microbiological control requires the maintenance of a recipient colony with a specific microbiological content that will determine the microbiological status of the litter [123]. A vasectomized colony to produce pseudopregnant females is also needed or alternatively, infertile genetically modified male mice [71, 124].
The recipient colony must be large enough for a caretaker or technician to be able to select appropriately the receptive females to be mated with a vasectomized male [71]. Approximately 50 to 75 females are required to be able to choose only 10 to 15 females in estrus and thus increasing the chance of having plug positive females [71]. Either inbred or outbred strains can be used as recipient females, although some strains tend to produce better results and facilitate transfer because of their large infundibula, such as CD1 mice [71].

Mouse pseudopregnancy

Pseudopregnancy induction and hormonal regulation

Mouse pseudopregnancy (PSP) is a hormonal state, similar to an early pregnancy, in response to a mechanical stimulus, at the cervico-vaginal area [17, 125-127]. This stimulus will result in a neuroendocrine response, which will last for several days and will cause the release of the hormone prolactin, essential for the CL maintenance and progesterone production [17, 125, 126].

The hormonal effect caused by the maintenance of a group of females without male stimulation, known as Lee-Boot effect, was characterized in 1955 [128] as a spontaneous PSP that occurred in females housed in the described conditions. This state of PSP was also described by Dewar in 1958 as having a duration of 14 days and characterized by weight changes, mammary gland development and absence of estrous cycles together with vaginal mucification [129]. PSP duration is variable, and values vary from 10-12 days [17, 125, 127] or the already referred 14 days.

PSP in mice is induced and hormonally regulated in a slightly different way when compared to rats. In rats it can be induced by several intromissions, without ejaculation, which will result in a biphasic secretion of prolactin [125, 126]. The biphasic secretion of prolactin occurs once during the day and a larger peak at night, during the first 10 to 12 days of pregnancy or PSP [125]. This prolactin biphasic secretion is essential to maintain the CL and consequently the progesterone levels. Memory pathways are proposed to be related to the prolactin release as ovarian steroids are not essential to keep the prolactin production pattern after cervical stimulation and it seems that this stimulus is kept as a memory for the prolactin surges during the first days of pregnancy or PSP [125, 130]. In mice, however, the mechanical stimulus is not sufficient and ejaculation is essential to induce PSP together with a certain number of intromissions [125]; females mated with sterile males that ejaculate are described as being all pseudopregnant. Unlike rats, this initial stimulus in mice does not induce a biphasic prolactin secretion. In mice, the mechanical and ejaculatory stimulus induces a prolactin surge around day 6 of pregnancy or PSP [125]. After mating, during the end of proestrus, beginning of the estrus, progesterone and estrogen levels increase, as a result of the LH and FSH surges. Prolactin surge will be important to maintain the CL as it has antiapoptotic and luteotropic actions and promotes the secretion of LH and progesterone, essential during pregnancy [17]. The secretion of progesterone will then be ensured by the placental lactogen, which will maintain the CL after day 11 to 12 of pregnancy or, the CL will be eliminated by increased prostaglandin F2α (PGF2α) and decreased progesterone levels [17].

Pseudopregnancy determination and optimization

For embryo transfer, PSP induction is essential, and several points can be considered to determine PSP prior to embryo surgical transfer. More commonly, PSP is determined by the presence of a vaginal plug in the morning after mating with a sterile male and, once the oviduct is exposed, the presence of a large ampulla, bloody fluid, small clots on the ovaries and also the presence of the cumulus mass around oocytes, all good signs that the female is in PSP [17, 71]. In the absence of these signs, PSP was probably not induced, and

another female should be considered for the transfer. Treatment with two doses of progesterone followed by mating on day 4 is described as an alternative method to the visual choice of receptive females (females in estrus or proestrus), that can significantly increase the number of plugs and reduce the size of the receptive females colony [131]. Without hormonal synchronization, the simple determination of the estrus cycle before mating, combining visual determination and vaginal smears [132] can also be used to increase the chances of plug positive females.
Females at 0.5 dpc are used for oviduct embryo transfer and females at 2.5 dpc are used for uterine transfer.

Mouse implantation and pregnancy maintenance

Once the transfer is performed, a fine tune between the embryo and the uterine horn and reproductive organs is needed for a well succeed implantation. The blastocyst is only able to implant during a short period of time, called implantation "window" [133, 134], during which stress factors can contribute to low implantation success, particularly during day 3 of pregnancy [135, 136]. The mouse uterus is considered to be receptive to implantation on day 4 of pregnancy [137] and by the day 5, the uterus is no longer able to accommodate implantation. After the pre-ovulatory surge, estrogen production starts to increase promoting proliferation of the uterine epithelial cells [134, 137, 138]. By the second day of pregnancy, the levels of estrogens decrease and apoptosis is installed in the uterine cavity; progesterone levels start again to increase by day 3 and together with a second preimplantation surge of estrogens, the conditions for implantation are set [134, 137, 139-141]. The combined action of progesterone and estrogens is essential to the implantation process. In fact, the implantation "window" can be prolonged by maintaining the progesterone levels without the estrogen preimplantation surge [134, 140]. An important feature that results from this balance between the levels of estrogen and progesterone is the microvilli flattening, a clear feature of uterine receptivity [142]. Several genes and factors are involved in the implantation process such as the leukemia inhibitory factor (LIF), signal transducer and activator of transcription 3 (STAT3), BMI1 and nuclear receptor coactivator 2 (NCOA-2) [139]. After epithelial flattening, other phases occur that will lead to implantation namely, the "attachment reaction" during which the blastocyst is in direct contact with the apical membrane of the uterine cells [142]. A third step of differentiation and decidualization induces the stromal cells surrounding the blastocyst to proliferate and differentiate into specialized cells called decidual cells [137]. During the next days of development, the production of placental lactogen and progesterone will replace prolactin for CL maintenance [126, 143]. In fact, placental lactogen and progesterone levels are directly proportional to

the number of fetus [144]. Adding to the already mentioned hormonal regulation, the state of the blastocyst is also an important factor for the implantation process as inactive blastocysts, which can be activated by estrogen induced factors in the progesterone primed uterus, are related to a shorter implantation "window" [145].
Many factors regulate the implantation process, from genes like Fkbp52, Hand2 to Msx1 and Klf-5 [137] which will not be explored as these are not the aim of this work.

Male mouse vasectomy and genetically altered mice alternatives

Two independent colonies are needed to perform surgical embryo transfer: a sterile male colony and the recipient female's colony.
Sterile males can be either obtained by producing surgically vasectomized males or by keeping a genetically modified mouse strain, from which, a part of the males can be used for PSP induction. The efficiency of this colony will affect the number of females as intromission and ejaculation are needed for PSP establishment.
Surgically induced vasectomized males are produced by removing a small portion of the *vas deferens* that connects the epididymis to the urethra and blocking the remaining ends with a surgical suture line. Males should be around two months of age and any strain with a good reproductive performance is suitable for this purpose [71]. Older males tend to have fat deposits around the inguinal area and vasectomy can be more challenging. Two surgical approaches can be used for this technique: an abdominal approach in which the *vas deferens* is exposed through a midline ventral abdominal incision and a scrotal approach where the same exposition is performed via the scrotal sac. Despite some authors recommended the use of the scrotal approach, considered to have a lower impact on animal welfare [146], other authors considered that a combined analgesia between paracetamol and a non-steroidal anti-inflammatory drug (NSAID) such as ketoprofen is determinant for the animal recovery and the differences in welfare indicators during the post-surgical period are not significantly different between animals submitted to the abdominal or scrotal approaches if efficient analgesia is provided [147]. After surgery, males should be tested but at least 1 week for recovery is recommended before mating these animals [71]. Keeping accurate records of breeding performance is important to efficiently manage the vasectomized colony.
Another alternative to vasectomized animals is the use of genetically modified strains whose males are either not able to produce sperm or produce deficient sperm. *Gapdhs*^{tmDao} mice are an alternative option for the production of pseudopregnant females. In these knockout homozygous males, whose reproductive behavior is normal, the glyceraldehyde-3 phosphate dehydrogenase spermatogenic gene was deleted and due to low adenosine

triphosphate (ATP) levels, their sperm has low motility [148]. This strain showed no significant differences when compared to surgically vasectomized males on plugging and pregnancy rates and frequency of offspring [148]. A transgenic model overexpressing Prm1-EGFP fusion protein is another genetically modified strain that can be used as sterile males [149]. Protamines (apart from being related to chromatin condensation, protection of the male haploid genome and suppression of transcription), are determinant for sperm shape [124]. The referred model is able to mate and produce plugs but are infertile due to the deficient spermatid formation and maturation, resulting in impaired motility and viability [149]. Testing these animals before use is also recommended.

Using genetically modified mouse strains to produce PSP has a significant impact on animal welfare as surgical procedures are avoided but a larger number of litters are needed to produce the same number of genetically modified infertile males when compared to the number of litters needed to obtain animals for vasectomy [148].

Surgical procedure for embryo transfer

Embryo transfer can be performed using embryos in different stages which can be transferred to the oviduct or to the uterine horn, the first requiring a female at 0.5 dpc and the second in 2.5 dpc.

Oviduct unilateral transfers require one incision of approximately 1 cm, parallel to the lumbar vertebrae, over the left or right flank. A second incision on the fascia beneath the incision is used to expose either the left or the right fat pad, and bulldog forceps are used to fix it and expose the ovary and oviduct. Once the oviduct is exposed, the surgical procedure can be performed under the microscope and the infundibulum is exposed through a small cut on the ovarian bursa. Embryos loaded inside a pipette are then inserted through the infundibulum and released inside the this tube, directed towards the ampulla [93]. The oviduct is then returned to the abdominal cavity and the fascia and skin closed with suture lines.

Bilateral transfers are very similar to the unilateral but, the initial incision is performed over the lumbar vertebral bones and this single incision will be used to have access to both sides of the abdominal wall and expose the two ovaries and oviducts.

A variation on this technique consists in performing a small cut before the ampulla, using spring scissors and inserting the pipette through this small cut, instead of using the infundibulum natural opening [71, 150].

Uterine transfers are also similar to the oviduct transfers but, after exposure of the ovary fat and ovary, a 30G needle can be used to produce a small opening in the uterine horn, close to the utero-tubal junction, and the pipette is inserted through this area [71, 93].

Anesthesia and analgesia are required for this procedure and will be discussed further on.

Factors affecting the efficiency of embryo transfer

Embryo transfer success is determined by the birth of a litter and its ability to survive during the next days. For rederivation purposes, the microbiological content and the elimination of specific pathogens is also a measure of success. Several variables are determinant for this success which is measured not only as an event of birth and survival of the litter, but also as the effort needed between the number of embryos used and the number of pups obtained after transfer. Pregnancy rates obtained after transfer are another measure of success for this procedure.

Surgical variations, unilateral and bilateral transfers and embryo number

The surgical technique used for embryo transfer is described as an important factor related to the success of embryo transfer. Different surgical approaches were already described in the previous topic from bilateral and unilateral transfers, uterine and oviduct transfers and two small variations on the way the pipette is inserted (infundibulum versus ampulla insertion). Only recently some work was published regarding unilateral and bilateral transfers, however, this factor seems to be closely related to the number of embryos used per transfer. Johnson *et al*, in 1996 [151], published the first insight on the optimum effort between the number of embryos used for transfer and the resulting number of pups born. This author concluded that transfers using 15 embryos from the B6SJLF1 strain or more did not result in higher number of implantation sites and fetus on the gestation day 19; however the number of live pups after birth, was not evaluated [151]. More recently additional work based on historical results of embryo transfers from genetically modified mice at a B6 background, concluded that transfers of low number of embryos (in this case 8 to 12 embryos) produce a better outcome on the number of pups born [96], using unilateral transfer via infundibulum. An additional paper described the efficiency of embryo transfer using similar number of two-cell embryos (15 to 18) after unilateral and bilateral transfer, concluding that bilateral transfers do not produce a significantly better outcome on the number of pups born [94]. This group also used historical data of genetically modified strains at a C57BL/6N or C57BL/6J background. More recently, in 2019, another group published data related to unilateral and bilateral transfer: historical results from genetically modified strains, from several different strains including C57BL/6, FVB and BALB/c, were compared for unilateral transfers using 15 or less embryos at a two-cells stage, also via infundibulum,

and bilateral transfers using more than 15 embryos [95]. Again, lower number of embryos produced higher success rates (between 10 and 20) and more than 21 embryos did not produce significantly better results but strain differences were also highlighted [95]. Together, these papers show that a low number of embryos tends to produce higher success rates, measured as the effort between the number of embryos transferred and the number of pups obtained. A number as low as 5 embryos can produce a viable litter [95]. Higher number of embryos transferred seem to be less efficient as more than 15 or 21, depending on the authors, do not produce significantly higher number of pups. Bilateral transfers are also described as not having a significant advantage as the number of pups is not much higher and it is a more complex and potentially painful procedure for the receptive female. These findings are however difficult to compare as different strains were used, many related to other genetical alterations that may affect the result, different strains of recipient females were also used and in one of the cases, the effective number of pups born was not evaluated so, comparing each of the variables in an independent way is not possible. However, we can definitely state that lower number of embryos seem to produce more efficient results.

Apart from using different surgical approaches related to bilateral or unilateral transfer, other small surgical variations are also described as possible sources of variation. Johnson *et al* compared transfers performed using the infundibulum, using a small cut (figure 3) or separation of the bursa involving the ovary, or doing a small hole just before the ampulla and no significant differences were found between the two techniques [151, 152]. Transfers using the infundibulum tend to be technically more challenging as the infundibulum can many times remain in a position with difficult visualization and bleeding is also common when this technique is used [152].

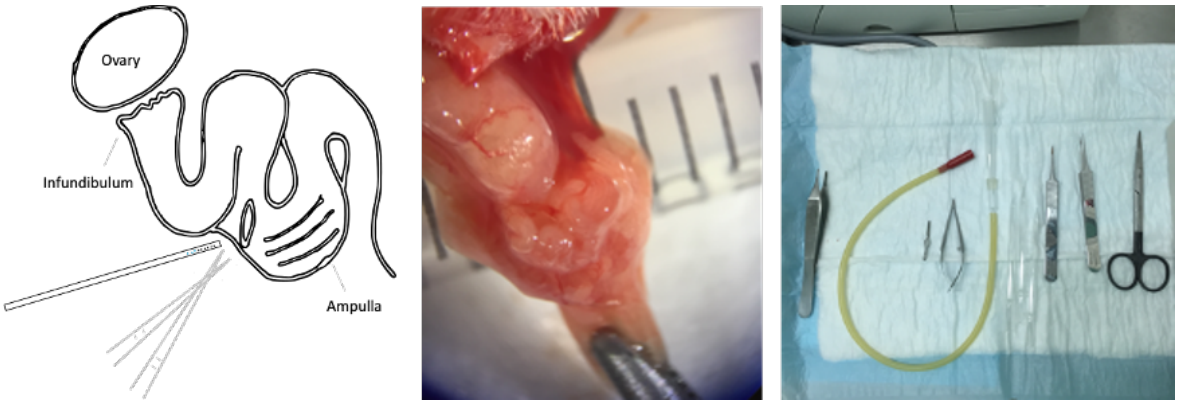

Figure 3: Mouse oviduct transfer using a small cut before the ampulla; mouse oviduct and surgical setup.

Pipette variations, calibration and medium

The type of pipette used for embryo transfer is another critical point to consider. Pipettes should have a relatively narrow tip, just large enough to accommodate the embryos and minimize the volume of medium; the tip must be smooth to prevent damage of the embryos or at the oviduct [71]. A modified pipette with a syringe like tip was described as having 20% higher success rates when compared to a conventional pipette, probably due to the smaller hole produced by the pipette that prevents embryos withdrawal after pipette removal [153]. Another modified pipette was also described, again with a syringe like tip attached to a micro pump, producing lower levels of embryo withdrawal after removal and pregnancy rates when compared to the conventional method [154].

The injection of air bubbles to prevent embryo withdrawal and to control the pipette capillarity may also affect the outcome of embryos transfer but these air bubbles are also helpful to detect medium movement during the transfer. The amount of bubbles should be minimized (two to three) as these can affect embryo implantation [71]. The injection of air bubbles into the uterine horns after blastocyst transfer results in higher frequencies of decidua and dead fetus [155] and it affects embryo spacing and post-implantation development [156].

The volume of medium used for embryo transfer should be minimized and M2 or other HEPES-buffered medium can be used for the transfer [71, 93]. As already described for incubation medium, its content will affect embryo development and survival. Reducing the medium allows for a minimal interference in the naturally existing oviduct fluids, optimal for embryo development. Supplementing the culture medium with urokinase plasminogen factor, plasminogen and IGF2 can improve implantation rates when blastocyst transfers are used [108] and supplementation with vascular endothelial growth factors and antioxidants produces similar results on implantation [111, 157]. Hyaluronan supplementation to the transfer medium is also described as having positive results on implantation and fetal developmental rates [158]. High glucose content medium, however, reduces the number of pups per litter, probably via activation of mechanisms that lead to an increased production of reactive oxygen species (ROS) and apoptosis [159].

Recipient female's factors and oocyte background

Embryo transfer can be performed using different receptive female's conditions: age, strain and estrous cycle at the time of transfer are important to consider as possible sources of experimental variation. Female's strain choice should consider the maternal skills and the anatomical features that facilitate the surgery [71]. Several strains were reported as

recipient females: CD1 [71, 94, 96, 160-162], (BALBxDBA)F1 [95], (B6xCBA)F1 [71], Swiss webster [163] and NMRI [164] mice are among the more common ones. Rose *et al* described transfers using NMRI, DBA/2J and C3H mice as recipient females and concluded that despite NMRI produced better results, DBA/2J or C3H females could also be used as recipient females, reinforcing the idea that strain related variability exists on what is concerned to the recipient female [164]. The survival of the embryo seems to be more dependent on the strain of the receptive female than on the embryo strain [165, 166] but the post-natal survival appears to be dependent on both the recipient female and the pup background [166]. The recipient strain effect on litter survival can be related to their maternal skills as some strains levels of cannibalism and infanticide tend to be higher, such as C3H/HeN and DBA/2J [164]. But this variability is not only related to the recipient female strain but also to the strain of the embryos used for transfer. DBA/2J are particularly challenging to use and the number of embryos needed to produce a viable litter is higher when compared to other strains (15 to 30 per female, using the bilateral technique) [162, 164]. BALB/c strains also tend to have lower success rates when compared to B6 strains [95] so the number of embryos transferred should be adjusted according to the strain.

Recipient female's age and weight can be optimized too. Females between 11 and 13 weeks were described as optimal for embryo transfer [167], although this age is strain dependent because different strains have different developmental rates and the amount of fat surrounding the oviduct will interfere with the easiness of the transfer. When using CD1 females, weight ranges between 25 to 35 g, corresponding to females at 6 to 8 weeks of age are recommended [71]. Older females also tend to produce lower success rates and heavier females higher success rates [168]. The diet fat content of recipient females will also be determinant for better pregnancy rates and lactation [169].

The synchronization between the female estrous cycle and the timing of embryo transfer has been described as a possible point of improvement as transfers of blastocysts during the diestrus produce higher implantation rates [170]. The synchronization between the embryonic phase and the uterine phase of pseudopregnancy seems to be a determinant factor. Transfers of blastocysts into the oviduct of 0.5 dpc pseudopregnant females are possible and when performed simultaneously with zygotes, the blastocysts remain in a dormant state and are activated latter, migrating rapidly for uterine implantation [171] but when blastocysts are inserted into the oviduct, low birthweight's are reported, showing that blastocysts are more sensitive to embryo transfer [172]. This ability to develop blastocysts and zygotes simultaneously in different oviducts reveals that each oviduct has the potential to independently regulate embryo development.

As a way to reduce the number of recipient females used, more than one embryo transfer per female has been suggested and described as having no effects on embryo transfer

outcome or animal welfare [173] but considerations should be made as the same animal is submitted to two surgical procedures and thus, the overall severity increases.

Anesthesia and analgesia considerations for embryo transfer surgery

Animal welfare during and after the embryo transfer surgical procedure must be ensured and a balance between the best anesthetic and analgesic regimen from a welfare point of view and the experimental outcome must be achieved.

Isoflurane anesthesia, avertin (tri-bromoethanol) and a mixture of ketamine and medetomidine or ketamine and xylazine are described as suitable for embryo transfer, without significative differences in this procedure outcome [174]. Isoflurane anesthesia has the advantage of producing a fast induction and recovery and is relatively safe to be used but requires a more complex setup and maintenance when compared to injectable options [174]. It was reported that isoflurane can be detrimental for embryo development when two-cells embryos are exposed to concentrations of 3 and 5% isoflurane, impairing the passage to blastocyst [175], however this effect was not identified when recipient females were anesthetized with isoflurane. Tri-bromoethanol has been described as having the potential to cause acute abdominal inflammation and diffuse to focal necrosis at the abdominal wall and, for that reason, its use for embryo transfer surgical procedures is not recommended [176], although its safety margin is higher when compared to ketamine and xylazine or medetomidine [174]. Recent work concerning embryo transfer used ketamine and xylazine or isoflurane, most commonly, as anesthetics [94-96].

As for analgesia, several options are described as having no negative effects on embryo development. Common options are NSAID's, such as carprofen and meloxicam [94, 95] that have the advantage of having long half-lives, allowing for a reduced number of post-operative administrations. Tolfenomic acid and flunixin meglumine are also options for analgesia in animals submitted to embryo transfer, and tolfenomic acid has been associated with better outcomes when compared to meglumine, with higher pregnancy rates and number of pups born [177]. Other options include paracetamol, buprenorphine, tramadol among others. The effect of the chosen analgesic must be efficient on eliminating post-operative pain but its effect on embryo development must be minimal. Paracetamol is a convenient drug with analgesic effects and the possibility of being administered through the drinking water allows for an important refinement. It has been described as not affecting pregnancy rates and the offspring weigh [178]. Opioids, such as tramadol, can be used as analgesic options as no negative effects on embryo transfer were reported [179]. Buprenorphine can be used alone or in combination with carprofen or flunixin meglumine,

without negative impact on embryo transfer [180-182]. Morphine, however, is not recommended as it impairs blastocyst implantation [183].

Mouse parturition and litter survival

The last step for a successful transfer is the birth of the litter and its survival.
Parturition is initiated by the fetus but is dependent on progesterone decrease [17]. The release of lung surfactant into the amniotic fluid initiates macrophages migration into the uterus and promotes an inflammatory like response that induces uterine contractility and the production of PGF2α [17]. Most female mice will give birth at 19.5 dpc when embryo transfer is performed [71].
A normal female behavior and ability to nurture the pups is essential for the litter survival. Embryo transfer can have effects on the offspring and the strain of both the mother and the pups may also affect their behavior and consequently, their survival.
Reciprocal and non-reciprocal transfers have shown that the mother genetic background plays an important role on embryo survival [164-166, 184]. Some strains, such as DBA/2J are related to lower litter survival after reciprocal or non-reciprocal embryo transfer, but better results are achieved when other strains are used as recipient females [164], such is the case of C3H females, who are able to produce larger pups [166]. Fetal growth is mainly dependent on the maternal and fetal genotype until mid-gestation and the fetal genotype will determine the gestation length [184].
Maternal behavior differences among strains are described and may influence pup survival. Differences between maternal behavior of C57BL/6J, 129Sv and Swiss mice related to nest building, licking, grooming behavior and interaction with the pups are present: 129Sv, as an example, enroll in a nest building behavior, engage with pups and show licking and grooming behavior less frequently than the other two strains [185].
Ultrasonic vocalizations are a communication tool between mother and pup determinant for pup survival and maternal behavior. These are used for a variety of purposes, such as calling the mother for retrieval.
On the other hand, embryo transfer will determine some behavioral characteristics on the offspring. Females resulting from embryo transfer were described as having reduced nest building and increased exploratory behavior and anxiety [186]. Female mice from NMRI, resulting from embryo transfer using a NMRI recipient female also presented a depression like phenotype [186].

Non-surgical embryo transfer

Non-surgical embryo transfer (NSET) is alternative to surgical embryo transfer that consists in inserting a blunt catheter through the vaginal opening, at 2.5 dpc, when the cervix is dilated, to allow the release of blastocysts into one of the uterine horns [93]. This method can be performed with a conscient female, without the need for anesthesia or analgesia and thus contributes for the 3R's application. This technique is related to less cardiac fluctuations and lower fecal corticosterone levels (stress indicators), when compared to surgical embryo transfer and, from a welfare perspective, is a good alternative [187]. Success rates are described to be similar to surgical embryo transfer, some authors referring to success rates of 66 to 74% [188]. Small improvements of the technique were also described such as the use of females from the strain B6CBAF1/OlaHsd as insertion in this strain is easier when compared to CD-1 females; to correctly identify females in PSP, using only plug positive females and choosing females at the ideal estrus cycle phase at the time of mating [189]. However, NSET can only be used to transfer blastocysts and consequently can be related to difficulties in culturing embryos up to a blastocyst stage or, in alternative, allowing the embryos to grow in utero after a superovulation protocol. Embryos cultured *in-vitro* also have a slower development and thus, transfer of blastocysts resulting from zygotes that are manipulated and then cultured up to the blastocyst stage tend to have lower success rates [189], which can explain the difficulties in implementing this technique.

Mouse rederivation and the microbiota manipulation

Embryo transfer as a microbiological control and standardization tool

Mouse embryo transfer, as already described, can be used in a context of ART, such as the case of embryo transfer after embryo manipulation or cryopreservation, or in a context of microbiological control and standardization. In both situations, the offspring microbiological content will be determined by the recipient mother. In fact, embryo transfer and cross fostering can be used as tools to manipulate the mouse microbiota [190].

Embryo transfer, cross-fostering and cesarean-section (c-section) are important methods to prevent microbiological agents in an animal facility, whose impact on animal welfare and health is very significative. Large scale rederivation can decrease mortality related to several diseases, such as lactation ileum, or weight loss in animals used for ENU related transgenesis [191]. Many microbiological agents impair animal health and affect growth curves, reproduction, overall survival and may confound experimental outcomes. Although

the cost of rederivation and microbiological control inside an animal facility can be high, the benefits related to this process outcome this limitation [191]. The techniques referred are most of the times used to create a specific pathogen free (SPF) status but the fact that SPF mice have low microbial diversity and their immune system is less robust when compared to a wild mouse or a pet-shop mouse, raises the question if these animals are representative models of disease [192].

The efficiency of the 3 methods described (embryo transfer, cross-fostering and c-section) is variable and embryo transfer, although technically more challenging, is more efficient at eliminating unwanted agents.

Cross-fostering consists in using a foster mother, with a similar age litter to the one to be transferred, which will adopt and nurse the transferred pups. For that, female's pregnancies are synchronized so that the litters are born in approximate dates. After birth, the pups from the foster mother can be reduced to allow a similar size litter and normal growth. For better results, the litter to be transferred can be mixed with bedding from the foster mother to prevent rejection [193]. The use of docile strains with good maternal skills is also key for a successful transfer. The timing of transfer after birth is determinant for the efficiency of this technique as a mechanism to eliminate unwanted agents. *Helicobacter hepaticus* , as an example, can be eliminated using cross fostering if transfer is performed in the first 24 hours after birth [194]. Changing the animals into a clean cage 24 hours before the birth and consequent cross fostering was also described as an efficient method to eliminate mouse norovirus (MNV), mouse hepatitis virus (MHV) and *Helicobacter* spp. but *Syphacea obvelata* can't be eliminated using this method [195]. Cross-fostering limitations are related to the fact that pups will be exposed to microbiological agents during the birth process, through the vaginal canal, and contact with feces, urine and oral and nasal secretions will also be impossible to prevent right after birth.

C-section is another alternative method for the elimination of unwanted agents and to define a specific microbiological content. To perform it, a pregnant female is euthanized at 18.5 dpc, when pups are sufficiently develop to survive outside the uterus [71]. After removal of the amniotic fluid and nasal secretions, pups are kept warm and then moved into the foster mother. Similarly to the cross-fostering, a foster mother with a newborn litter is required and gentle mix of the pups from the two mothers or reduction of the foster mother litter may be needed [71, 196]. C-section, although more efficient than cross-fostering on the elimination of agents, is related to significative alterations on the microbiota that are also related to a higher predisposition to disease. Metabolic diseases have been related to the use of c-section and mice born through this method are heavier than mice born via natural birth [197, 198]. The impact of c-section on the immune system is reflected on a decrease of T-cells and regulatory markers such as Foxp3 and IL-10 [199, 200]. An increase of invariant natural

killer T (iNKT) cells and macrophage markers was also related to c-section and despite the GM transfer to GF animals can recreate these alterations, the levels of T-cells can't be restored [199]. A higher incidence of diabetes type 1 [200, 201], allergies [202] and celiac disease [203] were also related to c-section.

The described techniques will shape the microbiota according to receptive or foster mother and have been used as tools to obtain a certain GM. The nursing mother related to cross fostering will determine the pups GM in a permanent way [204] and embryo transfer of CD-1 into different recipient females also resulted in a modulation of the GM whose stability was maintained for several generations [205].

The microbiota modulation and its impact on the phenotype of different animals has raised several questions regarding results reproducibility. A common problem in laboratory animals are the different responses to a specific protocol or phenotype variations between animals of the same strain. Wild type mice housed in different areas but with a same origin have GM variations that can affect experimental results [206]. Small differences in the GM were found in genetically modified mice from IFN-γ, recombination activating gene 1 (Rag1) or IL-4 KO strains housed in two different conditions (open cages and individually ventilated cages (IVC)) [207]. Experimental results are dependent on several variables and control of the GM or at least characterization of the GM is a recommended step to increase reproducibility [4].

The newborn immune system priming and disease predisposition

Prenatal and early life exposition to microorganisms is determinant for the immune system development in an irreversible way. This exposition will ensure a sufficient diverse GM that has been related as more physiological than a restricted GM. The GM of the mother will start shaping the fetus GM during pregnancy. An evidence of that is the SCFA passage through the placenta, as referred by Kimura *et al* [3]. Antibiotic administration to female mice during pregnancy seems to favor the growth of microorganism that produce less SCFA and consequently, the offspring of these mothers is more prone to obesity [3]. Asthma susceptibility has also been related to antibiotic treatment during pregnancy and consequent GM alteration [208]. A reduced microbial diversity and increased IgG and IgM was also described in antibiotic treated pregnant females [209] and increased levels of lymphocytes and low levels of interferon-gamma (IFN-γ) were detected in pups resulting from these mothers [209, 210].

Newborn mice will be exposed to the mother vaginal microbiota if a natural birth occurs and this early exposition will shape the GM of the pup. Children born through c-section, were related as having lower levels of *Bifidobacteria* and higher levels of *Clostridia* [211] and a

higher predisposition to allergic diseases. The GM of mice born through c-section is also less abundant in *Ruminococcus* and *Rickenelaceae* and several alterations on the immune system (described on the previous topic) were also detected [200]. This immune system priming occurs right after birth and will have a determinant influence on the maturation of this system and subsequent predisposition for the development of certain diseases. The birth mode will determine the way this process occurs. This “window” of time is limited as, later stimulation in life will not allow a proper immune system maturation [200, 212]. Colonization of the gut of GF mice at 3 weeks of age results in a pro-inflammatory state [213]. In fact, GF animals have an accumulation of iNKT cells in the colonic lamina propria that can be prevented by gut colonization right after birth but not if colonization occurs as adults [214]. Delayed colonization is equally characterized by low levels of IL-10, IFN-γ and transforming growth factor beta (TGF-β) [211].

The consequences of these early GM alterations were described by several authors and higher predisposition to obesity, allergic asthma, inflammatory bowel disease (IBD), colitis, atherosclerosis, rheumatoid arthritis, multiple sclerosis, type 1 diabetes, lupus, among others, have been described [2, 215-221]. Controlling the GM content of laboratory animals is, for the reasons discussed previously, essential to understand disease mechanisms and to ensure results reproducibility.

GM control and variation factors

Tools to shape the GM into a specific content and its limitations

Several tools can be used to establish a specific microbiota in order to allow the use of specific models of disease and to study the interaction between a group of microorganisms or a single organism and the phenotype of a given animal model.

Antibiotic treatment is a simple and easy method to change the GM, mainly when the target is the adult animal. Its effects will depend on the animal used, antibiotic spectrum, dose and time of administration. Several antibiotic regimens and cocktails are described to manipulate the GM [222] which makes comparison of results of antibiotic treated animals difficult. The antibiotic regimen will eliminate a group of bacteria but, at the same time, this can cause an imbalance on the remaining microorganisms and favor the overgrowth of certain bacteria [190]. This is a limitation when interpreting results of animals submitted to antibiotic treatment but other limitations must also be considered: antibiotics administered through the drinking water are related to dosing issues and palatability, which many times requires the addition of other reagents like sugar, although oral gavage, more labor intensive, can be used; effects in other areas outside the gastrointestinal tract (GIT) were

also described and must be considered [223]. Antibiotic's effects on myeloid and lymphoid cells are characterized by increases in eosinophils and basophils, at the same time that neutrophils, macrophages, monocytes and granulocytes levels decrease [223]. Th1 and Th17, as well as IFN-γ, tumor necrosis factor alpha (TNFα), and IL-17 are also decreased but the Th2 response (IL-4, IL-5 and IL-13) is increased, denoting a shift from a Th1 to a Th2 response [223]. It is also important to recognize that antibiotic effects lasting time is dependent on the type of antibiotic used. A cocktail using amoxicillin/metronidazole/bismuth produces alterations on the GM that are reversed after 2 weeks of treatment stop but the use of cefoperazone produces a much larger reduction of microorganisms and its effects are not completely reversed when the treatment finishes [224].

Fecal transplants are also commonly used protocols to recreate a specific GM. It can be used in GF animals or after a period of antibiotic treatment to reduce or deplete bacterial colonization [190]. This method, also used in humans, seems to be efficient on the treatment of *Clostridium difficile* [190]. Recently, the use of SPF animals treated with laxatives or antibiotics before fecal transfer was described as a valid alternative to the use of GF animals for fecal transplantation. Juvenile mice were referred as having a more similar GM to the initial donor and better engraftment results when compared to adult SPF animals and despite the fact that, 9 weeks after transfer, the GF animals GM was more similar to the donor one, SPF juvenile animals were considered a valid alternative to the GF mice [225]. The use of antibiotics or laxatives before transfer improved the engraftment result [225]. Apart from the initial GM, fecal transplants may also be determined by genetic differences that may play a role in the GM content of animals [190].

GF animals are often used for GM studies but the challenges for the maintenance of such animals are considerable and very specific conditions and technical knowledge are required to maintain this type of animals. These are very useful when studies of one specific agent (mono-association) are required or when a specific microbiological content is required, such as when creating gnotobiotics, however, the physiology of these animals is far from normal so, results must be interpreted with caution [190]. GF mice have enlarged cecum, less developed small intestine, impaired food absorption and also express high levels of glucagon-like peptide, related to the inhibition of food intake [226]. These animal's Peyer patches are in lower number and less developed and smaller mesenteric lymph nodes are also present [227].

Other animal models such as defined microflora or mono-associated are used for GM studies. These allow for the study of a single microorganism or a defined group of microorganisms, such as the case of the altered Schaedler flora (ASF). This a common group of 8 bacteria, used for years for microbiological studies [228], and allows for a normal mucosal immunity in mice and similar cecum sizes to regular mice [190]. This model, which

can also be used in rats, has a low complexity flora that is recognized as being similar to the mouse conventional flora and so explore in a simpler way the interactions between different microorganisms and the immune and other systems [228].

Humanized xenographted mice are a more translatable model that gives the opportunity to mimic the complex human GM through the use of fecal transplants. Although mice are more commonly used as a model for GM study, rats in fact have a more similar Firmicutes: Bacteroidetes ratio to humans and are able to maintain a more stable expression of the transferred microbiota than mice, but the lake of genetically modified models makes mice a more suitable model [227]. Models that can harbor the complex human GM are thus in high demand however, the recipient mouse genetics and the differences in the mucosal immune response and its influence to allow or avoid the maintenance of all the species originally transferred must also be considered as a potential limitation of such models [190]. GF animals are usually the recipients of these fecal transfers and the early neonatal interaction between the GM and the immune system is lacking in these animals. Transfers into conventional mice were also described and remain an alternative to the use of GF mice: the human fecal material transfer performed after bowel cleansing was kept for 4 weeks [229].

Other alternatives to the fecal material transfers include co-housing, involving the exposure of a certain animal to another animal with the desired GM. The main limitation of co-housing is the fact that it relies on a passive transfer of microorganisms and not all the species will be transferred using this method [190].

At last, cross-fostering, c-section and embryo transfer are also used for GM alteration and standardization. As already discussed, the 3 methods are mainly dependent on the GM of the receptive/surrogate mother but the timing since the maternal stimulus will start influencing the offspring GM varies according to the method [204, 205].

Microbiota influencing factors

Interpreting GM studies results must be cautious and precise control of experimental variables must be implemented as the GM is affected by different factors.

As already discussed, breeding strategies (rederivation, cross-fostering, c-section) do have an impact on the GM of mice and the advantages and disadvantages of each of these methods were already discussed from a microbiological point of view. Of noting that, rederivation is a very common tool for the safe introduction of animals inside a barrier facility.

An important source of GM variation is the diet. Diet alterations induce changes in the GM as fast as 1 week after the dietary change [230] and the diet composition will favor the

growth of certain microorganisms and consequently may lead to a state of homeostasis or dysbiosis. The production of SCFA and its beneficial effects on the host system is a recognized benefit of carbohydrates and fiber metabolization and its effect as a cancer preventive have also been described [231]. Starch and non-starch polysaccharides, which will act as prebiotics, will enhance the growth of *Bifidobacteria* and *Lactobacilli*, whose growth is also dependent on the protein origin [231]. Higher levels of protein promote the growth of Bacteroidetes but higher levels of carbohydrates promote the growth of Prevotella [2]. High sugar diets will also promote lower microbial diversity and low levels of SCFA and are related to increased severity colitis [232]. In some cases, the effect of the basal diet overcomes the effect of fecal transfers. As an example, animals receiving fecal transplants from lean and obese animals develop a distinct GM but, after receiving continuously a high fat diet, the GM content no longer relates to the donor phenotype [233]. It is, for the discussed reasons, advisable that the same batch of diet is used during the whole experiment.

The water source must also be considered as acidified or autoclaved water will interfere with the GM content [2] but other husbandry practices will also interfere: number of animals per cage, bedding material, cage type and recent transport [2, 190, 227]. The animal vendor is another important source of variation as reflected by the differences in GM content of animals from the same strain but with different origins but also differences that are vendor and strain dependent [234]. The abundance of segmented filamentous bacteria (SFB), believed to play a role in the intestine immune response [235], in animals with origin in specific vendors is an example of variability related to the GM according to the vendor [190]. Animal's age and gender are also sources of GM variation. Earlier exposition in life to microorganisms was already described as a determinant factor for the immune system development and GM establishment. In humans, the Firmicutes: Bacteroidetes ratio is variable according to the age [236] and in mice the GM characterization varies according to its age too, were different microorganism groups were identified in different phases of development [237].

As far as gender differences in the GM, these are described in non-obese NOD mice where females have a higher incidence of type 1 diabetes [238]. A study comparing 89 inbred mouse strains concluded that differences between sexes are present in many strains, particularly in C57BL/6J and C3H/HeJ [239]. Some immune system gender related differences were also described by Fransen *et al* [240]: bacteria like *Alistipes*, *Rickenella* and *Porphyromonadaceae,* known to be increased in the absence of immune system defenses, were increased in males and the transfer of male GM induced more inflammation, probably due to the presence of inflammation promoting bacteria [240]. The same study also concluded that the female GM is less efficient in preventing allergies, resulting in lower

RORγt^{+}Foxp3^{+} T cells expression in Peyer patches and in the mesenteric lymph nodes [240].

The genetic background role must also be considered when performed GM studies. The severity of some diseases in dependent on the genetic background of the animals, such as the case of the GPx1 and GPx2 double knockout whose ileocolitis severity is mild in a C57BL/6 background but severe in a 129S1/Sv genetic background due to the genetic predisposition to *Escherichia coli* in the later one [241]. The GM dependency on the genetic background was recently demonstrated using F1 hybrids of a cross between B6 and BALB/c mice, revealing that, despite the different GM contents of the parents, F1 hybrids presented similar GM [242]. In some cases, the genetic background effect is more pronounced than the GM effect as revealed by the different responses to high fat diet treatment in C57BL/6J and A/J, kept even after a fecal transplant between the two strains [243].

GM is also variable according to the gastrointestinal tract region [244], although frequently, the most commonly studied area is the colon as it allows for a non-invasive sample collection.

The gut immune system and the microbiota immunomodulation

The gut mucosal barrier

The mucosal barrier is an important component of the immune system with impact on the innate and adaptive responses. The gut mucosal barrier includes a series of structures and cells within the lamina propria such as antigen presenting cells, immature lymphoid cells, Peyer patches, CD4^{+} and CD8^{+} T cells and B cells [245]. The intestinal epithelial cells and the integrity of this layer is another important component of the gut mucosa, where Goblet cells produce mucus and Paneth cells produce antimicrobial peptides (AMP's) [246] contributing to the epithelial cell integrity and preventing a direct contact with potential pathogens. Intestinal epithelial cells are able to present antigens to dendritic cells (DC's), whose modulatory effect on T cells is essential to determine an adequate immune response. Together with macrophages, DC's are part of the innate immune response at the gut mucosa and impact intestinal homeostasis through the regulation of T cells responses. Several types of DC's are present at the lamina propria, mainly CD103^{+} DC's, CX3CR1^{+} DC's and plasmocytoid DC's [246]. Despite their slightly different functions, DC's bridge the recognition of microbial antigens and the B and T cells response at the PP and mesenteric lymph nodes (MLN) [246-248].

The DC's, together with the epithelial cells, the mucus layer, the IgA production, the immune cells and the antimicrobial peptides produced by epithelial cells are the so called "mucosal firewall" [247] which is highly dependent on the GM content.

Macrophages also play an important role in the gut barrier, secreting IL-10, with anti-inflammatory effects, and consequently, IL-10 will promote the proliferation of Treg's and reduce inflammation [246].

The mucus produced by Goblet cells prevents a direct contact between microbes and the epithelial cells or the lamina propria, which can lead to pathogenic events. Although the outer part of the mucus is colonized with microorganisms, the inner part is sterile [246]; deficiencies in the mucus layer, such as the case of mice with deficiency in core 1 synthase (C1Galt), responsible for the production of the mucus major component, leads to the development of colitis [249].

IgA production, driven by the action of DC's at the PP and MLN's, is not related to a classic memory response, which allows a constant adaptation to a dynamic GM [247]. The levels of IgA are an important marker of the interaction between the mucosa and the GM [250].

PP's in mice are mainly concentrated at the small intestine, whose cells ability to mount an IgA response is higher when compared to the colon, where a more tolerant directed response is predominant [250]. In fact, the T cell response in the colon is characterized by increased Treg's and lower levels of Th17 and the ileum response is richer in Th17 [250].

Apart from the mechanisms described below, AMP's produced by Paneth cells are also essential to create a proper immune response. In the small intestine, Goblet cells and thus mucus production, are reduced but AMP's such as regenerating islet-derived protein 3 gamma (Reg3) and α and β defensins contribute to the separation between the bacterial content and the epithelial cells [246].

Several cytokines are present at the gut environment, such as IL-10, TGF-β and other molecules such as vitamin A and retinoic acid [248]. Retinoic acid has an important role in the induction of T-cells, inducible Tregs and in the suppression of Th17 cells [248].

The balance between the different T cells response, mainly Th1, Th2, Th17 and Tregs is a key point to regulate the immune response. The Th1 response is predominantly a pro-inflammatory response whose main cytokine is IFN-γ and is oriented towards intracellular bacteria and viruses; the Th2 response is characterized by the increased IL-4, IL-5 and IL-13, production of IgE and is directed to extracellular bacteria and parasites [251]. On the other side, Th17 cells produce IL-17, Il-21, IL-22 and its roles include neutrophils mobilization and enhancement of TNFα, IL-6 and IL-8 production, having so a pro-inflammatory action [251, 252]. The Th17 response, under the balance of Treg's [252] is important in host protection against bacteria and fungal invasion but these cells are also

involved in dysregulated environments such as lupus and asthma; in fact, the Th17 response is related to divergent cytokine profiles [253].
Treg's and DC's are two essential elements for gut homeostasis. Natural Treg's prevent autoimmunity and inducible Treg's depend on antigen stimulation from antigen presenting cells and suppress immune responses in chronically inflamed tissues [248]. The modulatory effect of commensals is mainly dependent on the induction and expansion of Treg's, that reduce inflammation through IL-10 and TGF-β [248]

The interaction between the mucosal immune system and the GM

The GM and the mucosal cells are in constant interaction and their impact on the immune response is significative. The maturation of the immune system and its normal development it's dependent on the early exposition of the gut mucosa to a healthy microbiota.
The GM promotes the intestinal epithelial cell layer integrity, produces essential nutrients and metabolizes indigestible components of the diet, enhances the intestinal flora diversity, inhibits the attachment and proliferation of pathogens and participates in the control of potentially pathogenic agents [247, 248, 254, 255]. The GM will also modulate the immune response and educate it towards a moderate response [255].
The role of the GM in the immune system modulation is patent in GF animals whose reduced and underdeveloped PP's and MLN, low T cells levels, low IgA production and AMP's and high IgE levels are reverted by colonization with a healthy GM [255, 256]. Th17 cells are also reduced in GF animals and the development of this cell type is dependent on the colonization with SFB. SFB are able to promote epithelial cells to produce serum amyloid A and reactive oxygen species (ROS) which in turn are related to an increase in Th17 [246] and also increase the production of IgA [246, 247]. In fact, the colonization of GF animals with a human GM is not enough to revert the altered immune response but the transfer of human GM + SFB does restore the T cells levels [257].
The colonization of the gut is essential to modulate the immune response but a clear differentiation between commensals and pathogenic agents is needed at birth. For that to happen, a state of tolerance is needed to allow the colonization by a healthy GM after birth. TLR tolerance after birth is low [253] and it allows commensals to be settled at the intestinal tract [247]. Later in life, the TLR activation by commensals will inhibit exaggerated inflammatory responses and is key for homeostasis. TLR's recognize microbe-associated molecular patterns (MAMP's), such as lipopolysaccharide (LPS), flagellin or muramic acid, and shape the response of the immune cells, activating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and the release of cytokines and chemokines [253].

The influence exerted by commensal microorganisms in the immune system can occur through cellular components of bacteria, such as polysaccharides [248]. Polysaccharide A produced by *Bacteroides fragilis* induces Treg's expansion and production of IL-10, limits Th17 response conferring protection of *Helicobacter hepaticus* associated colitis [247]. Commensal microorganisms also limit the growth of pathogenic microbes via production of antimicrobial compounds like bacteriocins and linoleic acid [248] or by promoting the production of antimicrobial peptides by epithelial cells [253].

The production of SCFA is another example of the impact microorganisms can have on the immune system modulation. SCFA (mainly butyrate, acetate and propionate) are used as an energy source for intestinal epithelial cells and determine the action of DC's, macrophages and cytokine production [256]. These metabolites also participate in Treg's differentiation and function [247, 256]. The production of IgA is equally stimulated by SCFA, preventing the adhesion of bacteria to the epithelial cells [245]. The balance between different bacteria is determinant for the production of SCFA: increased ratio of Bacteroidetes/Firmicutes after treatment of mice with high fiber diet increased the levels of SCFA and prevents the development of Th2 mediated allergic inflammation in the lung [258].

Dysbiosis and the reflects of GM shifts on the immune system and disease predisposition

Dysbiosis and shifts in the microbiota have been linked to immune system dysregulation or inadequate responses and predisposition to certain diseases, including cancer. It is likely that most of the pathological changes are not related to one single microorganism but to the balance between several microorganisms and consequently alterations in the signs produced by commensals that shape the immune system [247, 255]. One example is the case of *Clostridium difficile*, that only causes disease in case of depletion of commensals, usually related to antibiotic therapy [251].

Common causes of dysbiosis include antibiotic therapy, stress and high protein diets [259]. Dysbiosis can lead to the activation of Th17 and neutrophils and, consequently, increases inflammation [245].

In cases of IBD, there is reduced bacterial diversity and an increased prevalence of Proteobacteria [260] as well as other bacterial shifts [252]. Patients with IBD are also characterized by an excessive Th17 response, defective Treg cells, increased IL-17 and IL-22 and reduced TGF-β, IL-10 and IL-33 [252] illustrating that dysbiosis can direct the immune system towards a pro-inflammatory state.

Gut microbiota and gastrointestinal cancer development

The microbiota and dysbiosis have been related to several types of cancer, not only gut associated cancer but also other cancers such as lung, gallbladder, melanoma or pancreatic cancer, among others [261, 262]. This relationship between cancer and the microbiota is based on the modulation that the microbiota is able to do regarding inflammation and host immune system response and can either be tumor protective or the opposite. Moreover, the microbiota modulation can be used in several cancer therapies from breast cancer, head and neck or colorectal cancer (CRC) [263] and the efficiency of immunotherapy such as the case of immune checkpoint blocker's (ICB's) is also dependent on the GM [251, 256, 264].

Gut associated tumors, and particularly CRC, are related to initial hyperplasia that results in colonic epithelial proliferation and loss of epithelial normal architecture giving origin to a benign polyp [261, 265]; this polyp can also invade the mucosa and submucosa and become an adenocarcinoma.

Inflammation is closely related to cancer development as demonstrated by the increased risk of CRC development in inflammatory bowel disease patients [261]. The GM is, as already stated, essential for the development of cancer: GF animals have a reduced incidence of tumor development and GF $APC^{Min/+}$ mice also develop very low number of tumors, despite their genetic predisposition [261].

The already described immune gut system and the recognition of MAMP's by pattern recognition receptor's such as the TLR's are essential to determine the host immune response and are also related to the development of cancer, as the activation of this system leads to the initiation of an inflammatory response characterized by increased IL-17, TNF-α, IL-6 and activation of NF-kB and STAT-3 [262]. The inflammatory process is not only important for tumor initiation but also for tumor growth as tumor infiltrating myeloid cells also produce pro-inflammatory cytokines that promote proliferation and reduce apoptosis [262].

The GM, either related to dysbiosis or to the presence of pathogenic microorganisms, is able to promote carcinogenesis via multiple strategies. Several bacteria have developed strategies that allow it to escape the host immune response and survive. Polysaccharide rich capsules, as an example, limit the complement activation and other molecules/structures such as lipopolysaccharide, flagella and peptidoglycans and can refrain the host immune response [266]. These mechanisms can, apart from allowing bacterial survival, also induce carcinogenesis. One example is the case of CagL, an adhesion protein of *Helicobacter pylori* that upregulates gastrin production and consequently can promote carcinogenesis [266].

Toxins and ROS produced by bacteria can promote carcinogenesis through direct DNA damage, triggering cell death resistance signaling and promoting proliferation [266]. *E. coli* can produce colibactin through the polyketide synthase (pks) and induces DNA breaks [266, 267]. Bacterial toxins can also promote signaling pathways inducing proliferation and resistance to cell death. Enterotoxic *Bacteroides fragilis* (ETBF) activates the Wnt/ β-catenin and NF-kB pathways promoting inflammation and cell proliferation [253, 266]. ETBF is also able to induce Th17 cell infiltration triggering tumor growth [265]. *Fusobacterium nucleatum*, on the other hand, is able to use an adhesin (FadA) to activate the Wnt/ β-catenin and NF-kB pathways and at the same time is able to escape the NK cells action [265].

Other oncogenic bacterial mechanisms are related to the ability of some bacteria, like Salmonella, to take the control of intracellular components such as the phagosome, and influence signaling pathways related to proliferation and apoptosis [261].

Bacteria metabolites can equally trigger carcinogenesis: secondary bile acids, hydrogen sulfide production and ammonium are all related to carcinogenic effects [262]. Secondary bile acids result from the metabolization of primary bile acids by intestinal bacteria and lead to carcinogenesis, for example through the production of ROS, inducing DNA damage; hydrogen sulfide impairs the use of butyrate by colonocytes, reduces mucus production and increases colonocytes proliferation, as already stated [262].

How the GM can promote antitumoral effects and improve cancer therapy

Some therapeutic strategies are highly dependent on the GM to be efficient and the use of antibiotics or dysbiosis caused by other treatments have the potential to interfere with drug efficiency.

Oxaliplatin is an example of a chemotherapeutic drug that is dependent on the GM to be efficient. This drug inhibits DNA synthesis, induces cytotoxic and antitumor activity and it is also dependent on the production of ROS to produce DNA damage and apoptosis [268]. However, treatment with antibiotic or a decrease in the diversity of the GM reduces the production of ROS and consequently the efficiency of oxaliplatin [264, 267, 268].

Cyclophosphamide is another example of a drug whose therapeutic activity is dependent on the GM. This drug promotes damage in the intestinal mucosa and the translocation of bacteria (*Lactobacillus johnsonii* and *Enterococcus hirae*) into the mesenteric lymph nodes, inducing the production of Th17 and Th1 cells with antitumoral activity [251, 253, 264]. Antibiotic treatment or GF animals with tumors treated with cyclophosphamide have an impaired production of Th17, reducing the treatment efficacy [269].

The efficiency of immune checkpoint inhibitors is also dependent on the GM. Immune checkpoint inhibitors act by reactivating T cells for antitumoral response, such as the case

of programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibitors [270]. The probable cause for the GM impact on its efficiency is the cross reactivity between tumor antigens and bacterial antigens: the transfer of *Bacteroides fragilis* and Bifidobacterium overcomes the blockage in the therapeutic that exists in GF or antibiotic treated animals with CTLA-4 or PD-1 respectively [264].

Immune response against cancer cells and its dependency on the GM

Cross reactivity between tumor antigens and bacterial antigens has been proposed as a mechanism used by the host to fight tumor cells and represents a relatively new and significant target for cancer therapy. Tumor antigens are related to low immunogenicity and thus do not trigger a robust immune response, however, tumor neoantigens are highly immunogenic and can provoke a T-cell mediated immune response [271]. Accordingly, the GM is determinant for the definition of the immune response, either towards a tolerance response or a pathogenic one and it is also accepted that the GM has an impact on the immune response triggered by neoantigens [264, 271].
The recognition of neoantigens shared between bacteria and tumor cells is the basis of some immunotherapy as already discussed.

$CD8^+$ T cells: antitumoral effects and microbiota dependency

$CD8^+$ T cells, cytotoxic T lymphocytes (CTL's), play an important role towards intracellular pathogens and also seem to be an ideal target for cancer treatment [272]. The role of these cells is shaped by the microbiota and dysbiosis seems to promote a pro-tumorigenic action [273]. CTL's can be related to pro-tumorigenic and anti-tumorigenic roles. These cells are primed by DC's and NK cells and exert a pro-inflammatory action using two main mechanisms: granules exocytosis (perforin production will allow the introduction of granzymes A and B able to disrupt the cancer cell machinery) and induction of apoptosis mediated by FasL [274]. CTL's release IFN-γ and TNF-α, and in turn, IFN-γ stimulates the differentiation and action of M1 macrophages to exert antitumoral responses [274]. TNF-α will also promote tumor cell apoptosis and TGF-β release, but at the same time can have a pro-tumorigenic action by reducing the CTL's infiltration and the activity of NK cells due to the expression of PD-1 and CTLA-4 [274]. The continuous exposure to IFN-γ can also lead to CTL's exhaustion [274]. The polarization of the immune response favoring Treg's and M2 macrophages will create an immunosuppressive environment and dysfunctional CTL's [274]. The role of CTL's is apparently dependent on the presence of specific bacteria. CTL's are reduced in mice after antibiotic treatment and also in GF mice [272]. A group of 11

bacteria were identified as being related to the induction of T cells and production of $CD8^+$ T cells, whose presence improved the response to anti-PD1 treatment and suppressed tumor growth [272].

The VCMSH2$^{LoxP/LoxP}$ mouse model

Lynch syndrome results from mutations in DNA mismatching repair systems (MMR), mainly the *Msh2* gene [275]. Although a knock out (KO) model was available for the study of MSH2, this model commonly developed lymphoma and thus its utility for the study of Lynch syndrome was limited and a LoxP model with the MSH2 dysfunction only at the intestinal cells was created, the VillinCreMSH2$^{LoxP/LoxP}$ mouse [275]. This mouse model is characterized by tumor development mainly at the small intestine, a large number of microsatellite instability at the intestinal cells and lesions present somatic Apc (adenomatous polyposis coli) mutations [275]. This model is frequently used in combination with the mutations at the *Apc* gene, tumor suppressor gene that regulates the Wnt/ β-catenin pathway, inducing increased number of polyps in the small intestine at an early age [276]. Most patients with MMR mutations develop colorectal cancer, suggesting that this deficiency is, at least partially, related to the intestinal microenvironment, although the reason is not clear [276].

The gut microbiota also determines the phenotype of these mice. APC$^{Min/+}$ mice have reduced microbial diversity before tumor formation and the administration of antibiotic results in increased number of tumors [277]. A relationship between the gut microbiota and the development of intestinal polyps was described in APC$^{Min/+}$MSH2$^{-/-}$, where treatment with a cocktail of ampicillin, metronidazole, neomycin and vancomycin, resulted in a 10000 fold reduction on the microbiota and reduced number of polyps at 6 weeks of age [276]. This group also described that the levels of inflammatory cells and mutations were similar between treated and non-treated animals but a diet reduced in carbohydrates also produced a similar result regarding polyp reduction [276]. In fact, diet and the gut microbiota reduction induced by antibiotic treatment resulted in a decrease frequency of Firmicutes, mainly *Clostridiaceae, Lachnospiraceae and Ruminococcacea*, butyrate producing bacteria, that in high concentrations is able to induce epithelial cell proliferation, modulates the Wnt/ β-catenin pathway and induces an increased incidence of tumors [276]. Butyrate, and the GM, is able to dysregulate the Wnt/ β-catenin pathway, responsible for the proliferation control of epithelial cells at the crypts, leading to an aberrant accumulation of β-catenin [276]. The expression of β-catenin assumes again a nuclear localization if a low

carbohydrate (and consequently low butyrate) or antibiotic administration are performed [276].

Research goals

The main goal of this work was to further characterize ART techniques commonly used laboratory mice facilities with focus on the reduction of animal use to optimize the outcome of these techniques and to evaluate the possible impact of one of these techniques on the phenotype of a mouse model of intestinal cancer. For that, the following tasks were performed:

- Different schedules and ages were characterized for the outcome of superovulation using C57BL/6J animals. Weight and estrous cycle were also considered as variables for this experiment and the number of oocytes was determined in the described conditions;
- Historical data of GMM was analyzed concerning incubation and timing of embryo collection as a tool to increase the number of two-cells obtained and reduce the number of donor females;
- Embryo transfers using historical data and a set of controlled experiments was performed using two strains: C57BL/6J and B6129F1. Different number of embryos, unilateral and bilateral transfer, and reciprocity were tested in a way to optimize litter size and success rates;
- The VCMSH2$^{LoxP/LoxP}$ mice phenotype was compared inside and outside the facility barrier, mimicking SPF and conventional conditions. For that, inflammation levels were evaluated using fecal lactoferrin, histopathology, immunohistochemistry and 16S rRNA assays were performed.

Chapter 1

Harvesting of mouse embryos at 0.5 dpc as a tool to reduce animal use: data from C57BL/6J, B6*129 and FVB/NJ strains.

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Harvesting of Mouse Embryos at 0.5 Dpc as a Tool to Reduce Animal Use: Data from C57BL/6J, B6*129 and FVB/NJ Strains

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Abstract

Superovulation is used to stimulate the production and release of large amounts of oocytes in mice by using two hormones that mimic FSH (PMSG) and LH (hCG) effects. Since superovulation can have a negative impact on oocyte and embryo development, this investigation aimed to compare two alternatives for 2-cells embryo collection in order to reduce the number of females and to benefit from the superovulation process. Data from mouse embryo collection from our facility was analyzed to compare the number of 2-cells embryos collected at 1.5 dpc and the number of 2-cells embryos obtained after overnight incubation of 1-cell embryos, collected at 0.5 dpc. Genetically modified mouse strains with a similar background (C57BL/6J, B6*129 and FVB/NJ) were analyzed and for strains at a C57BL/6J and B6*129 background, the number of 2-cells embryos obtained after incubation was significantly higher when compared to the number of 2-cells embryos collected at 1.5 dpc (1.4-fold and 1.7-fold, respectively). C57BL/6J wild type mice had similar results with a higher number of 2-cells embryos when collection was performed at 0.5 dpc followed by incubation (1.4-fold). These results can help the planning of 2-cells embryo harvesting by reducing the number of females needed for this procedure.

Keywords

0.5 Dpc Embryos, 1.5 Dpc Embryos, Incubation, Superovulation

1. Introduction

Assisted reproductive techniques are widely used in the laboratory animal field, mainly in mice and rats, as a tool to improve reproductive performance and to reduce animal use. Superovulation is a technique used to increase the number of oocytes that each female ovulates by injecting pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) at an interval of 46 - 48 hours to mimic the effects of FSH and LH, respectively [1]. This technique greatly reduces the number of embryo donor females needed for a given purpose and has been optimized by controlling several factors that can affect its efficiency such as the female's weight [2] [3], age [4], hormone dose [2], interval between hormones administration [2] and estrous cycle phase at the time of hormone administration [5]. For rederivation purposes, superovulation is used to increase the number of oocytes released by each female that can then be collected after natural mating at 0.5 dpc (1-cell embryos) [6], at 1.5 dpc (2-cells embryos) [7] or even at 3.5 dpc (blastocysts) [8] for subsequent embryo transfer. Embryo transfer can then be performed at several stages of embryo development using 1-cell embryo [6] [9], 2-cells embryos [6] [7] [9] or blastocysts [8] [10]. Embryo culture can also be part of the process by collecting embryos at 0.5 dpc and cultivating up to a 2-cells or blastocyst stage.

For cryopreservation, collection of embryos at different phases can also be a possibility after superovulation [11]. Both superovulation and embryo culture have advantages and disadvantages. Although superovulation can improve the efficiency of embryo collection by synchronizing females and reducing the number of animals for a given purpose, it can affect oocyte and embryo's quality. Superovulation has been associated with a negative impact on oocytes and embryos by provoking changes in maternal and paternal imprinted methylation [12] [13] [14] and delaying embryonic and fetal development [14] [15] [16] [17] [18]. It has also been associated with alterations in essential proteins involved in regulation and translation of maternally stored mRNA with short poly A tail, such as poly(A)-binding protein (Epab) and poly(A)-binding protein cytoplasmic 1 (Pabpc1) [16] [19]. Important genes can be dysregulated due to superovulation, such as the Mest gene or the Grb10 [20] [21] and regulatory proteins can also be changed by this procedure like STAT3, leptin, transforming growth factor β-2 among others [22].

Allowing the embryos to grow *in utero* after a superovulation protocol can attenuate the effect of superovulation by decreasing the number of implantation sites [23], reducing the number of living fetuses [16] [18] as well as increasing pre-implantation mortality [14]. PMSG and hCG stimulation causes changes in the uterine environment that negatively affect embryo and fetal development [18] [24], resulting in reduced litter size and foetal organ's weight [25] [26]. These alterations include changes in lipid metabolism [27] and in NK cells [28], whose regulatory effects on implantation can be compromised. Alterations in the placental blood supply and on genes related with glucocorticoid regulation seem to be one of the responsible mechanisms for the decreased pup's weight [25].

Despite the superovulation disadvantages, this is still an important and needed

technique for the collection of large amounts of embryos or oocytes.

Embryo culture causes alterations on gene expression patterns of mouse embryos [29] and on the regulation of transposable elements (TE) and M16 media, frequently used for culture, has been associated with an upregulation of these when compared to KSOM [30].

Using embryo culture before the final application, either cryopreservation or embryo transfer, can have practical advantages such as the fact that only fertilized embryos are transferred into a pseudopregnant female or the possibility of splitting the work session in two or more days.

Literature regarding the best time point for mouse embryo collection is sparse and the timing of embryo collection after superovulation and transfer varies between articles. Van der Auwera [23] shows that when embryos are kept longer inside a superovulated oviduct, the number of implantation sites is lower. However, this was only described in a F1 hybrid CBAxC57BL6 background and no information was available regarding the number of embryos harvested at different time points after superovulation.

Since superovulation and incubation of embryos from different genetic backgrounds are common procedures in rodent animal facilities, and there are no standard protocols regarding the time for embryo collection after superovulation, this study may provide useful information about embryo harvesting.

The herein study aimed to explore the possible reduction on the number of embryos collected after a superovulation protocol during the early phases of development if embryos are kept *in utero* up to a 2-cells stage when compared to collecting the embryos at a 1-cell stage and allowing the passage in the incubator to a 2-cells stage. For that, we analyzed data related to embryo collection from historical data of genetically modified strains at different genetic backgrounds obtained between January 2017 and May 2019 at the i3S Animal Facility and data from C57BL/6J wild type mice.

2. Materials and Methods

2.1. Animals

The experiments were performed at the i3S animal facility and were reviewed and approved by the internal Animal Ethics Committee and by the Portuguese Competent Authority (DGAV) (project reference 2017_03, June 2017). Animals were housed at the i3S animal facility under standard conditions: temperature was kept between 20˚C and 24˚C and humidity between 45% and 65%; donor females were housed in groups of 3 to 4 females in type II eurostandard filter top cages (370 cm^2 floor area) for superovulation and then mated with a male, also housed in type II eurostandard cages (males were housed individually during the experimental period); food (2014 Teklad global 14% protein rodent maintenance diets, 2014S, Envigo Spain) and water (type II distillated) were supplied *Ad libitum*; bedding material was corncob and enrichment was also provided with a shelter or card tube and paper for nesting material. Animals were free from the following agents: MHV, EDIM, MPV, MVM, PVM, Sendai virus, TMEV, Ec-

tromelia, LCMV, MAD-1 and 2, Mouse cytomegalovirus, Reovírus, *Bordetella bronquiseptica, Citrobacter rodentium, Clostridium piliforme, Corynebacterium kutsheri, Cryptosporidium, Mycoplasma, Pasteurella spp, Pseudomonas aeruginosa, Salmonella spp, Helicobacter spp, Staphylococcus aureus, Streptococcus pneumoniae, Streptobacillus moniliformis*, *Klebsiella pneumoniae,* ectoparasites (*Myobia, Radfordia and Myocoptes*), *Eimeria, Entamoeba, Giardia, Spironucleus muris, Tritrichomonas, Aspiculuris tetraptera and Syphacea obvelata and muris*. Occasional positives were found at quarantine for *Pasteurella spp, Helicobacter spp, Kebsiella pneumoniae* and *Staphylococcus aureus* as animal origin differs among the two experiments, as explained on the next section.

Data herein presented arose from the collection of mouse (*Mus musculus*) embryos either for the purpose of embryo transfer (rederivation) or cryopreservation sessions, from January 2017 to May 2019. Data from embryo transfer and cryopreservation sessions are not stated as the aim of this work was to unravel the timing of embryo harvesting. A total of 312 sessions of embryo collection were included in the analysis. Data was grouped according to the genetic background and represent 37 mouse strains of genetically modified mice at a C57BL/6J background, 11 at a mixed background between B6 and SV129 and 6 at an FVB/NJ background housed at the i3S Animal Facility. Another set of experiments was also analyzed using wild type C57BL/6J animals (a total of 2 sessions of embryo harvesting). Animal origin was the i3S animal facility (in case the data correspond to embryo harvesting from cryopreservation sessions) or other facilities in case animals were in quarantine (for rederivation purposes). In that case, in quarantine, animals were mated one week after arrival and the litters from that breeding couple were used for superovulation and mating. For each session of GMM, donor females between 3 and 6 weeks of age from each colony were used. Although superovulation tends to work better in younger females, some females at 6 weeks of age were also used in all groups (harvesting at 0.5 and harvesting at 1.5 dpc) due to the availability of animals and to the fact that females at this age are also described to be used for superovulation [2]. For the C57BL/6J wild type group, females were between 3 and 4 weeks of age. Fertile males (minimum 8 weeks and up to 5 months old) were used. A ratio of 1 female for 1 male was used for mating and males were only used once per week.

2.2. Superovulation

PMSG (PMSG, HOR 272, ProSpec) and hCG (hCG, HOR 250, ProSpec) were diluted at a dose of 50 IU in sterile water and frozen at −20˚C until further use. Before injection, aliquots were thawed and diluted in sterile water to get a 100 μL dosage of 5 IU for intraperitoneal injection.

PMSG and hCG were given at an interval of 48h. At the time of hCG injection, females were mated with males overnight and checked for plug in the following morning. Only females with plug were considered for the data analysis to ensure that only oocytes with the possibility of being fertilized were being screened and fertilization was not a determinant factor on the result.

2.3. Embryo Collection

For 1-cell embryo collection, females were euthanized the morning after the mating (0.5 dpc) by cervical dislocation. Oviducts were collected in M2 media [31] and digested in M2 supplemented with 0.5 mg/ml hyaluronidase (Hyaluronidase, H4272, Sigma Aldrich) for less than 1 minute. After digestion, embryos were washed in 50 µL M2 drops, followed by several washes in 50 µL KSOM drops (EmbryoMax® KSOM Mouse Embryo Media, MR-020P-5F, Sigma Aldrich) and incubated in 50 µL drops covered with mineral oil (Mineral oil, M8410, Sigma-Aldrich). A maximum of 50 embryos per drop was incubated at 37˚C and 5% CO_2. In the following morning, the total number of 2-cells embryos was counted and embryos were then used for cryopreservation or embryo transfer. The initial number of 1-cell embryos was also counted before incubation.

For 2-cells embryo collection, females were euthanized by cervical dislocation at 1.5 dpc. Oviducts were collected in M2 media and flushed with the same media through the infundibulum, using a stereomicroscope (Stemi 2000 C, Zeiss) and a 30G needle. Two-cells embryos were then counted and washed in M2 media before cryopreservation or embryo transfer on the same day. For the wild type (C57BL/6J) session, the number of non-fertilized embryos obtained at 1.5 dpc was also counted.

2.4. Statistical Analysis

Data was analyzed using GraphPad Prism software (version 6.00 for Mac, GraphPad Software, La Jolla California USA). A D'Agostino & Pearson omnibus normality test was used before comparing 2-cells embryos, plug number and fertility rates. For the GMM strains, data were grouped according to the genetic background of each strain and a Mann-Whitney test was performed to compare the number of 2-cells embryos obtained from females with the same genetic background after incubation or after collection at a 2-cells stage. A Mann-Whitney test was also used to compare the mean number of plugs in each background in sessions using incubation and 2-cells embryos harvesting. For the C57BL/6J wild type session, a t-test was used to compare the mean number of 2-cells embryos and fertility rates. Data are shown as Mean ± SEM and $p < 0.05$ was considered as a statistically significant difference.

3. Results

Cryopreservation and rederivation are techniques that rely on collection of multiple embryos to successfully preserve a mouse strain or transfer to a recipient foster female. We started by collecting the data obtained from rederivation and cryopreservation between January 2017 and May 2019 from the genetic backgrounds most widely used in our animal facility: C57BL/6J, mixed background between C57BL/6J and SV129 (B6*129) and FVB/NJ. The number of sessions per genetic background, the number of genetically modified strains and the number of females used in total are summarized in **Table 1**.

With this data, we aimed at assess whether incubation of 1-cell embryos could

improve the number of 2-cells embryos obtained for each strain. A statistically significant increase of 1.4-fold ($p < 0.0001$) in the number of 2-cells embryos obtained after incubation of 0.5 dpc embryos when comparing to 2-cells embryos collection for C57BL/6J background (Figure 1(a)) was found. Similarly, an increase of 1.7-fold ($p < 0.0019$) in the number of 2-cells embryos obtained after incubation for strains at a B6*129 background (Figure 1(b)) was observed. For strains with FVB/NJ background, an increase of 1.6-fold in the number of 2-cells embryos was observed after culture when comparing to 2-cells embryos collection (Figure 1(c)) although this observation was not statistically significative ($p = 0.0698$). The mean number of plugs between sessions with the same genetic background was not statistically different for strains with a C57BL/6J and B6*129 background ($p = 0.3560$ and 0.5547 respectively). For FVB/NJ background, the mean number of plugs was significantly higher at the incubation group ($p = 0.0438$). Results are summarized in Table 2 and Table 3 for the harvesting at 0.5 dpc followed by incubation and 2-cells harvesting groups, respectively.

Table 1. Number of sessions and females analyzed for the different backgrounds from GMM.

	Collection at 0.5 dpc		Collection at 1.5 dpc		Number of GMM strains
	Number of sessions	Number of females	Number of sessions	Number of females	
C57BL/6J	64	299	183	926	37
B6*129	12	60	31	167	11
FVB/NJ	13	97	9	43	6

Number of sessions analyzed for the different backgrounds and number of females used in the total number of sessions according to the method of 2-cells harvesting: collection at 0.5 dpc followed by incubation and collection at 1.5 dpc.

Table 2. Data from GMM for 0.5 dpc followed by incubation groups at different backgrounds.

Background	Mean number of plugs per session ± SEM	Mean number of 1-cell embryos ± SEM	Mean number of 2-cells embryos ± SEM	Mean fertilization rates (%) ± SEM
C57BL/6J	4.672 ± 0.2826	22.72 ± 1.383	13.23 ± 0.9228	59.96 ± 2.331
B6*129	5.000 ± 0.5222	25.16 ± 3.351	16.86 ± 2.716	66.76 ± 5.689
FVB/NJ	7.462 ± 0.8444	19.88 ± 3.853	10.02 ± 1.867	58.88 ± 8.099

The mean number of 1 and 2-cells embryos per session is based on the number of embryos obtained divided by the number of females used per session.

Table 3. Data from GMM for 1.5 dpc harvesting groups at different backgrounds.

Background	Mean number of plugs ± SEM	Mean number of 2-cells embryos ± SEM
C57BL/6J	5.070 ± 0.2350	9.3 ± 0.7463
B6*129	5.3870 ± 0.3953	9.078 ± 1.731
FVB/NJ	4.778 ± 0.8625	6.377 ± 1.666

The mean number of 1 and 2-cells embryos per session is based on the number of embryos obtained divided by the number of females used per session.

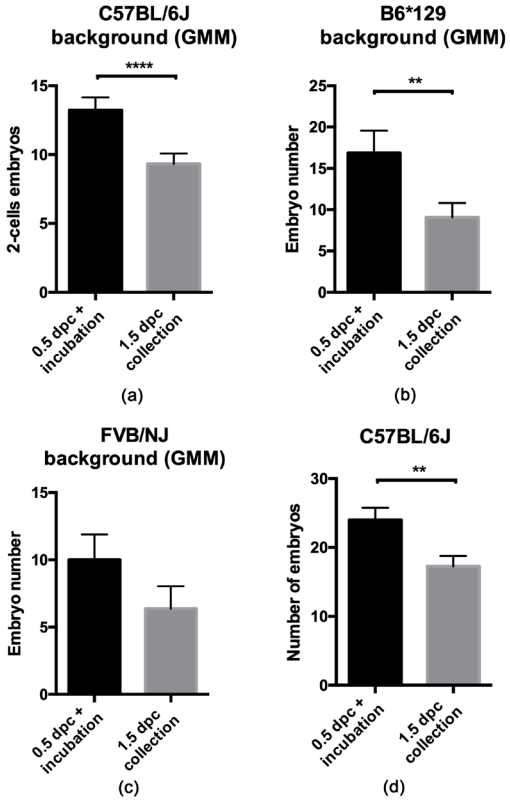

Figure 1. Mean number of 2-cells embryos obtained from collection at 0.5 dpc followed by incubation and 1.5 dpc collection at different backgrounds. Mean number of 2-cells embryos obtained from GMM or wild type strains using collection at 0.5 dpc followed by incubation and 1.5 dpc collection for: (a) C57BL/6J background (GMM); (b) B6*129 background (GMM); (c) FVB/NJ (GMM) background; and (d) C57BL/6J wild type. Results are expressed as Mean ± SEM.

When analyzing data obtained through the collection of 0.5 dpc followed by incubation and harvesting at 1.5 dpc from wild type animals (C57BL/6J), the number of 1 and 2-cells embryos was counted for both groups. A significant difference ($p = 0.0093$) was found between the mean number of 2-cells embryos collected at 1.5 dpc and at 0.5 dpc followed by incubation. The mean number of 2-cells embryos had an increase of 1.4-fold when incubation of 0.5 dpc embryos is used. No significant difference was found between the two groups fertilization rates ($p = 0.1898$). Data are summarized in Table 4 and Figure 1(d)).

The results herein presented suggest that, to obtain 100 fertilized embryos, we can reduce the number of superovulated females for the majority of the GMM strains analyzed, if the embryos are collected at 0.5 dpc and culture to 2-cells stage. For C57BL/6J, B6*129 and FVB/NJ strains are required 40%, 47% and 46% less females, respectively (Table 5). For C57BL/6J wild type animals, similar results are found. In this strain, incubation after 0.5 dpc collection leads to about 33% less females (Table 5).

Together, these results reinforce that, at least for the backgrounds analyzed, incubation of 1-cell stage embryos can increase the number of 2-cells stage embryos, thus, reducing the number of superovulated females.

Table 4. Data from C57BL/6J for 0.5 dpc followed by incubation and 1.5 dpc harvesting groups.

Collection at 0.5 dpc				Collection at 1.5 dpc			
Mean number of 1-cell embryos ± SEM	Mean number of 2-cells embryos ± SEM	Fertility rate (%) ± SEM	Number of females	Mean number of non-fertilized embryos ± SEM	Mean number of 2-cells embryos ± SEM	Fertility rate (%) ± SEM	Number of females
41.50 ± 3.122	24.00 ± 1.770	59.88 ± 3.742	22	8.813 ± 1.285	17.25 ± 1.529	67.09 ± 3.674	16

The mean number of 1 and 2-cells embryos was obtained individually, per female.

Table 5. Number of females needed for the collection of 100 embryos using the two harvesting methods for GMM at different backgrounds and wild type mice from the C57BL/6J strain.

		Collection at 0.5 dpc	Collection at 1.5 dpc
GMM	C57BL/6J	7	12
	B6*129	6	12
	FVB/NJ	11	20
Wild type	C57BL/6J	4	6

4. Discussion

Superovulation is a technique used to increase the number of oocytes produced and released by female mice. It has the advantage of decreasing the number of females needed for the collection of oocytes or embryos, contributing to the application of one of the 3R's. Despite the potential of this technique, superovulation has many times an uncertain outcome because the number of oocytes released by the female can be low [2] [4] [5] and also the quality of the oocytes or embryos produced after the injection of high doses of PMSG and hCG can be reduced [12] [13] [14] [16] [17] [18] [21] [22] [24] [27] [32] [33]. Our results show that, at least in strains with a C57BL/6J, B6*129 or with an FVB/NJ genetic background (although this last one was not statistically significant), it is more effective to collect embryos at a 1-cell stage (0.5 dpc) and culture to a 2-cells stage when compared to collecting embryos at a 2-cells stage (1.5 dpc) after female's superovulation. Similar results were achieved by Van der Auwera *et al.* [23] that considered that the oviductal *milieu* after superovulation have a negative impact on embryo development when compared to a non-stimulated oviduct or to *in vitro* culture using B6XCBA donors and NMRI pseudopregnant females. Collecting the embryos at the 1-cell stage can significantly increase the number of embryos obtained and thus, reduce the number of animals required for this type of protocols. The effect observed on C57BL/6J, B6*129 and FVB/NJ can be related to the fact that, after superovulation, embryo development is compromised and it is possible that removal of embryos from the uterine environment decreases the negative effect of superovulation by decreasing the exposition to PMSG and hCG. Taken into account that PMSG has an half-life of 40 to 120 h and the half-life of hCG is about 24 - 36 hours [27], a possible explanation for our results are the lower levels of PMSG and hCG exposition once embryos

are removed from the uterine environment and incubated in KSOM after several washes in M2. GMM strains with an FVB/NJ background didn't produce a statistically significant difference but this can be related to the smaller sample size available for this strain. Using a wild type strain such as the C57BL/6J, the same tendency of reduction in the number of 2-cells embryos when 1.5 dpc harvesting is used was noticed. The number of 2-cells embryos collected after incubation or even at 1.5 dpc is higher when compared to the same method using data from GMM strains at a B6 background. This can be explained by the fact that females used in the C57BL/6J experiment were all at an optimal age (3 - 4 weeks), resulting in higher number of oocytes produced after superovulation and also because this experiment resulted from only one wild type strain, having so, less variability. Nevertheless, thorough investigation will be needed to clarify the reasons why the number of 2-cells embryos is higher when incubation is used and to identify the changes observed on gene expression, lipid metabolism or the role of other essential mechanisms for embryo growth which are changed when superovulation is used and comparing it at a 1-cell and at a 2-cells stage. It would also be interesting to compare these results with embryos collected from females that are naturally mated, without superovulation. Our results are, however, compatible with those described by Van der Auwera *et al.* [23], reinforcing that the superovulation impact occurs not only at a later stage of development but also that the embryo reduction occurs soon after superovulation.

Genetic alterations among the strains used may also contribute to variable responses to superovulation, fertilization levels [34] [35] and incubation rates. Fertilization rates can be easily determined when embryos are collected at 0.5 dpc by considering the total number of oocytes/1-cell embryos and allowing passage to a 2-cells stage or detecting pronucleus under the microscope. However, when the harvesting is performed at 1.5 dpc, the total number of embryos counted (including non-fertilized and fertilized embryos) is lower, as shown in **Table 4**, suggesting that part of the embryos might be reabsorbed during the night. For that reason, the number of 1-cell embryos/oocytes obtained at 0.5 dpc and the number of non-fertilized embryos collected from the C57BL/6J wild type group were not compared but fertilization rates were not significantly different ($p = 0.1898$). This allow us to conclude that the effect observed on the reduction of the number of 2-cells when harvesting is performed at 1.5 dpc is not related to lower fertilization rates. For the GMM strains, the number of plugs was not different among groups and the fertilization rates for the 0.5 dpc groups was similar to the fertilization rates obtained with the wild type strains. Further data would also be needed to determine if this effect also occurs in other commonly used mouse strains.

5. Conclusion

Mouse superovulation can be optimized by controlling factors such as the female age, weight, dose and time of hormone administration. Our results show, that at least for the strains studied, a better planning of experiments involving the use of

2-cells embryos through incubation technique can significantly decrease the number of females needed to obtain a specific number of embryos. Thus, when no specific stage of embryo development is required (such as for embryo transfer for rederivation or cryopreservation), harvesting embryos at 0.5 dpc may provide a better outcome in the number of embryos than collecting embryos at 1.5 dpc (2-cells stage embryos). This practice may display a very positive impact on the reduction of animals used for superovulation contributing actively to the 3R's goals.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Chapter 2

C57BL/6J mouse superovulation: schedule and age optimization to increase oocyte yield and reduce animal use

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C57BL/6J mouse superovulation: schedule and age optimization to increase oocyte yield and reduce animal use

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Abstract

Superovulation protocols have been described for different mouse strains, however the numbers of animals used are still high and still little information is known about hormone administration schedules and estrous cycle phases. In this study, we aimed to optimize a superovulation protocol by injecting 5 IU of pregnant mare serum gonadotropin followed by 5 IU of hCG 48 h later, using three different schedules related to the beginning of the dark cycle (3, 5 and 7 pm) in a light cycle of 7 am to 7 pm, with light on at 7 am. C57BL/6J mice at 3, 4 and 5 weeks of age were used and the estrous cycle phase for times of PMSG and hCG injections was also analyzed. Total oocyte number was counted in the morning after hCG injection. Hormones given at 3 weeks of age at 3 pm (59 ± 15 oocytes) and 7 pm (61 ± 10 oocytes) produced a significantly higher oocyte number compared with oocytes numbers collected from females at the same age at 5 pm ($P = 0.0004$ and <0.0001 respectively). Females at 4 and 5 weeks of age produced higher numbers of oocytes when superovulated at 7 pm. No statistical differences between females at different phases of the estrous cycle were found. These results showed that in C57BL/6J mice, hormones should be given at 3 or 7 pm for females at 3 weeks of age, however older females should be superovulated closer to the beginning of the dark cycle to reduce female mouse use and increase the numbers of oocytes produced per female.

Introduction

Superovulation of female mice is still a common technique for production of large numbers of oocytes. The increased production of genetically modified mice over recent years with the introduction of the CRISPR/Cas9 technique has also been reflected by a high demand for mouse 1-cell embryos. Superovulation allows synchronization of females and the release of large numbers of oocytes, therefore reducing the numbers of animals needed for this purpose. Most protocols use two hormones to mimic the effects of endogenous follicle stimulating hormone (FSH) and luteinizing hormone (LH) through the injection of pregnant mare serum gonadotropin (PMSG) followed by human chorionic gonadotropin (hCG), with an interval of 42–48 h between the two hormones. Several factors affect the efficiency of superovulation and, therefore, the number of females needed for a given purpose. Female age (Gates, 1956; Gates and Bozarth, 1978; Hoogenkamp and Lewing, 1982; Sugiyama *et al.*, 1992; Redina *et al.*, 1994; Luo *et al.*, 2011; Kolbe *et al.*, 2015) and strain (Gates and Bozarth, 1978; Luo *et al.*, 2011), hormone dose (Gates and Bozarth, 1978; Legge and Sellens, 1994; Luo *et al.*, 2011; Wu *et al.*, 2013), interval between hormones administration (Luo *et al.*, 2011), the light cycle (Braden, 1957; Behringer *et al.*, 2016) and the mouse estrous cycle phase at the time of PMSG injection (Lim *et al.*, 1985; Tarín *et al.*, 2002) seem to be the most important factors.

Several reports have stated that, in C57BL/6J females, the optimal age for superovulation occurs before 48 days of age, at 21–32 days (Hoogenkamp and Lewing, 1982; Kolbe *et al.*, 2015). This finding was further supported by an alternative study that referred to a body weight of 14.2 g or less, corresponding to approximately 28 days of age, to be optimal to achieve the highest superovulation efficiency in C57BL/6NHsd mice (Luo *et al.*, 2011). In other strains, similar results have been described, with a tendency for better results with younger females, except in Crl:CD1(ICR) mice (Luo *et al.*, 2011; Kolbe *et al.*, 2015; Behringer *et al.*, 2016). Age and weight are important factors related to female onset of puberty and first ovulation. Juvenile females' sensitivity to negative feedback from endogenous estrogen decreases as puberty initiates and, consequently, FSH and LH levels rise, and the first ovulatory cycle begins. The onset of puberty has been related to vaginal opening, an apoptosis-mediated process dependent on the gene *Bcl2* (Mayer *et al.*, 2010). More precisely, the onset of puberty seems to occur 7 days after vaginal opening, when using the Pub-Score (Gaytan *et al.*, 2017). For better results, superovulation

should be performed before puberty, at 3–4 weeks of age or before vaginal opening (Kolbe *et al.*, 2015), occurring between 24 to 30 days of age (Fox *et al.*, 2006, Caligioni, 2009).

Attempts to optimize hormone doses and interval between hormones have been described previously (Legge and Sellens, 1994; Luo *et al.*, 2011), with a 5 IU dosage at an interval of 47–49 h to be optimal for most mouse strains. The estrous cycle at which the hormones are injected also affects the efficiency of the protocol. One study (Hogan *et al.*, 1986) stated that gonadotropins should be synchronized with the female estrous cycle to achieve optimal numbers and quality of pre-implantation embryos, plus higher percentages of oocytes that reach blastocyst stage when PMSG is given in estrus, diestrus-2 or proestrus.

The light cycle is also an important factor as it affects the time of LH release. hCG should be given 2–3 h before the endogenous peak of LH. In mice, the LH surge occurs 15–20 h after the midpoint of the second dark cycle (Hogan *et al.*, 1986). Legge and colleagues (Legge and Sellens, 1994) for mature mice (6–8 weeks old) described the impact of gonadotropin injection time in relation to the beginning of the light cycle. However, information is scarce regarding timing of administration in younger mice, which are commonly used for oocyte collection.

The aim of this work was to determine the impact of PMSG and hCG time of injection at different schedules in relation to the dark phase (3, 5 and 7 pm, and lights on between 7 am to 7 pm), using C57BL/6J mice of different ages (pre-puberal) and taking into account weight and estrous cycle phase at time of PMSH and hCG injections. Optimization of superovulation protocols remains an important tool in the effort to reduce numbers of animals used in several types of experiments and to allow maximum yield of oocyte numbers. Hormone doses were used based on results previously described (Luo *et al.*, 2011).

Materials and methods

Animals and husbandry

All experiments were approved by the i3S Animal Ethics Committee and Portuguese Competent Authority (DGAV) (Ref 2017_03), and performed at the i3S Animal Facility. Animal care was provided according to the European Directive 63/2010 and Portuguese Legislation. This facility is AAALAC accredited and follows the recommendations of the Guide for the Care and Use of Laboratory Animals, as well as FELASA recommendations. C57BL/6J female mice ($n = 89$) were used at 3–5 weeks old, weighing between 11 and 19 g, as this was the optimum age for superovulation (Luo *et al.*, 2011; Kolbe *et al.*, 2015). Animals were produced at the i3S/IBMC animal facility and breeders were replaced every other year using animals from the original colony to maintain a stable genetic background. Females were kept in standard conditions: temperature inside the rooms was 20–21^{0}C and humidity was 45–65%. Animals were fed with Envigo Teklad 2014S and provided with distilled water *ad libitum*. Females were housed in groups of five or six animals in type III Eurostandard cages (820 cm^2 floor area) with corn cob (Scobis Duo, Mucedola, Italy) as bedding material. Environmental enrichment was provided for all cages (card tube rolls and nesting paper) and animals were placed under a microbiological control programme, free of pathogens according to FELASA specific pathogen-free (SPF) recommendations. The light cycle corresponded to a 12 h : 12 h, dark : light period, with lights turned on at 7 am. All experiments were performed between September and December. Although some facilities use a 10 h : 14 h light cycle, most of articles describing superovulation describe a light cycle of 12 h : 12 h. Moreover, a 12 h : 12 h light cycle is the cycle adopted by the i3S Animal Facility and we aimed to obtain results that could be easily implemented within our current practices. Animal welfare was monitored during the whole experiment. Female estrous cycle was not synchronized before PMSG and hCG injection.

Experimental groups

In total, 89 females were used for this experiment and only females with an oocyte number higher than 1 were considered for statistical analysis. The 3 pm group contained 31 females, the 5 pm group had 27 females and the 7 pm group had 31 females. Each schedule group had females at 3, 4 and 5 weeks of age. Details regarding the number of females used for each of the administration schedules and age are stated in Table 1. Female weight was also registered and analysis was performed considering the weight and weight class. For the weight class, data were grouped as: less than 10.4 g; between 10.5 and 14.2 g; between 14.3 and 16.2 g and more than 16.3 g, as described previously by another group (Luo *et al.*, 2011). The number of females in each of the administration schedules according to weight class is represented in Table 2.

Superovulation

PMSG (ProSpec, HOR 272) and hCG (ProSpec, HOR 250) were diluted at a dose of 50 IU in sterile water and frozen at −20°C until further use. Before injection, aliquots were thawed and diluted in sterile water to prepare a 100-µl dose of 5 IU. Both hormones were injected by intraperitoneal injection. Vaginal swabs were stained with Diff Quick (907-1073, Henry Schein, Spain) and classification of the estrous cycle was carried out based on the method described by Caligioni (2009). On the day of the beginning of the experiment, a vaginal swab was collected 30–60 min before PMSG injection. PMSG was administered at 3, 5 and 7 pm and 48 h afterwards, a second vaginal swab was collected, 30–60 min before hCG administration, followed by this last hormone administration.

Oocyte analysis

At 12–14 h after hCG injection, females were euthanized by cervical dislocation. The number of oocytes was counted individually. For that, each oviduct was collected and placed in M2 medium. Hyaluronidase (Sigma Aldrich, H4272) was used at a concentration of 0.5 mg/ml in M2 and each oviduct was teared in a 100-µl droplet and kept for less than 1 min to allow cumulus cell removal. The number of oocytes within the cumulus cells and damaged/degenerated oocytes were counted using a Leica stereomicroscope (MDG41) at ×60 magnification. Due to the low numbers of degenerated oocytes, this variable was not considered in the final statistical model.

Statistical analysis

Statistical analysis was performed using SAS/STAT software. A general linear model (GLM) was used to compare the number of normal oocytes (dependent variable) collected assuming the following variables: schedule, age, the estrous cycle phase at the time of PMSG and hCG injection and weight either as a covariable or class (independent variables). Once the significant variables were determined, the GLM was repeated using only the significant terms. The final model was then:

Table 1. Mean number of oocytes and degenerated oocytes using the three different schedules of hormone administration based on female age

	Schedule of hormone administration					
	3 pm		5 pm		7 pm	
Age	Normal oocytes	Degenerated oocytes	Normal oocytes	Degenerated oocytes	Normal oocytes	Degenerated oocytes
3 weeks	59 ± 15 ($n = 6$)	9 ± 2	34 ± 9 ($n = 13$)	0 ± 1	61 ± 10 ($n = 8$)	0 ± 0
4 weeks	26 ± 9 ($n = 14$)	2 ± 2	32 ± 14 ($n = 9$)	1 ± 1	52 ± 10 ($n = 15$)	0 ± 0
5 weeks	25 ± 9 ($n = 11$)	2 ± 2	24 ± 7 ($n = 5$)	2 ± 2	38 ± 16 ($n = 8$)	0 ± 0

Values are mean ± standard deviation.

Table 2. Mean number of oocytes in the three different schedules, based on female weight class

	Hormone administration schedule		
Weight class (g)	3 pm	5 pm	7 pm
10.5 to 14.2	16 ± 0 ($n = 1$)	35 ± 12 ($n = 13$)	56 ± 9 ($n = 20$)
14.3 to 16.2	22 ± 8 ($n = 8$)	33 ± 13 ($n = 3$)	40 ± 16 ($n = 7$)
>16.3	37 ± 17 ($n = 22$)	27 ± 9 ($n = 11$)	40 ± 17 ($n = 4$)

Values are mean ± standard deviation.

Table 3. Mean number of oocytes based on estrous cycle phase at the time of PMSG and hCG injection

Administration schedule	Estrous cycle phase at PMSG	Estrous cycle phase at hCG	Mean oocyte number ± SEM
3 pm	Proestrus	Proestrus	24 ± 9
		Estrus	34 ± 19
		Metestrus	29 ± 0
	Estrus	Proestrus	40 ± 25
		Estrus	60 ± 21
	Metestrus	Proestrus	33 ± 8
		Estrus	40 ± 3
	Diestrus	Proestrus	22 ± 6
		Metestrus	26 ± 15
5 pm	Proestrus	Proestrus	32 ± 12
		Estrus	25 ± 6
	Estrus	Proestrus	30 ± 6
		Estrus	20 ± 0
		Metestrus	14 ± 0
	Metestrus	Proestrus	43 ± 10
		Metestrus	31 ± 17
7 pm	Proestrus	Proestrus	49 ± 16
		Estrus	53 ± 4
	Estrus	Proestrus	54 ± 4
	Metestrus	Proestrus	52 ± 14

$$y = \text{schedule} + \text{age} + \text{PMSG} + \text{hCG} + \text{schedule} \times \text{age} + e;$$

where 'y' represents the oocyte number; 'schedule' the hormone administration time; 'PMSG' the phase of the estrous cycle at the time of PMSG administration; 'hCG' the phase of the estrous cycle at the time of hCG administration; 'schedule × age' the interaction between these two factors and 'e' the model residuals. A *P*-value less than 0.05 was considered as statistically significant.

Results

Statistical analysis showed that weight (either as a continuous variable or when considered as weight classes) was not a significant factor for the number of oocytes collected ($P = 0.476$ for weight and 0.290 for weight classes). The estrous cycle phase at the time of PMSG and hCG injection was also not a significant factor for the number of oocytes ($P = 0.248$ and 0.9717, respectively, for estrous cycle at time of PMSG and hCG injections). Results for these two variables are represented in Table 2 (weight class) and Table 3 (estrous cycle), although these terms in the model were not significant. Schedule ($P < 0.001$) and age ($P < 0.001$) were considered as statistically significant factors, as well as the interaction between age and schedule ($P = 0.026$).

The mean number of oocytes and standard deviation (SD) are described in Table 1 based on female age and time of administration. The number of degenerated oocytes is also stated in Table 1.

The mean numbers of oocytes from the 3 pm group was 37 ± 2; at 5 pm the mean number of oocytes was 29 ± 3 and at 7 pm it was 50 ± 3. A significant difference was found between all groups, but the highest difference was obtained between embryos collected at 3 pm and 7 pm ($P = 0.009$) and between embryos collected at 5 and 7 pm ($P < 0.001$); significant differences were also found between 3 and 5 pm ($P = 0.026$). For age, the mean number of oocytes for females at 3 weeks of age was 52 ± 3; at 4 weeks was 36 ± 3 and at 5 weeks of age was 30 ± 3. A statistically significant difference was found between 3 weeks of age and 4 and 5 weeks ($P < 0.001$); no statistical differences were found between the number of oocytes collected from females at 4 and 5 weeks of age ($P = 0.085$). Interaction between age and schedule was considered to be statistically significative ($P = 0.026$). At 3 pm, the mean number of oocytes collected from females at 3 weeks of age was statistically different from the number of oocytes collected using the same schedule for females at 4 and 5 weeks of age ($P < 0.001$); the mean number of oocytes between females at 4 and 5 weeks of age at 3 pm was not significantly different ($P = 0.836$). At 5 pm, the mean number of oocytes was not significantly different between the three ages used (*P*-values between 0.123 and 0.392). The 7 pm group revealed a significant difference between the mean number of oocytes collected from females at 3 and 4 weeks of age ($P = 0.034$), between 3 and 5 weeks ($P = 0.0002$) and between 4 and 5 weeks ($P = 0.029$). Females at 3 weeks produced significantly more oocytes when superovulation was performed at 3 pm and 7 pm when compared to 5 pm ($P = 0.0004$ and <0.0001) but no difference was found for females at 3 weeks of age at

Table 4. Female's synchronization according to the administration schedules

		Female number and % at PMSG injection	Female number and % at hCG injection
3 pm	Proestrus	14 (45%)	17 (55%)
	Estrus	6 (19.4%)	10 (32%)
	Metestrus	4 (13%)	4 (13%)
	Diestrus	7 (22.6)	–
5 pm	Proestrus	11 (41%)	21 (77.8%)
	Estrus	10 (37%)	3 (11.1%)
	Metestrus	6 (22.2%)	3 (11.1%)
	Diestrus	–	–
7 pm	Proestrus	22 (71%)	29 (93.5%)
	Estrus	2 (6.5%)	2 (6.5%)
	Metestrus	7 (22.6%)	–
	Diestrus	–	–

3 and 7 pm ($P = 0.791$). At 4 weeks of age, the number of oocytes was statistically different between females superovulated at 3 pm and 7 pm ($P < 0.0001$) and at 5 and 7 pm ($P = 0.0001$) but not between 3 and 5 pm ($P = 0.591$). At 5 weeks of age, significant differences in the number of oocytes were found between females whose hormones were given at 3 and 7 pm ($P = 0.02$) and between 5 and 7 pm ($P = 0.039$) but not between oocytes collected at 3 and 5 pm ($P = 0.762$). The percentages of females in each phase of the estrous cycle are listed Table 4.

Discussion

Superovulation is a valuable tool to produce large numbers of oocytes using a reduced number of animals. Optimization of superovulation protocols is essential to maximize the use of such animals, which is an ethical obligation of all scientists using animals. Our results showed that females at 3 weeks of age, superovulated at 3 pm or 7 pm (4 h before the dark cycle or at the beginning of the dark cycle, respectively) produced significantly higher numbers of oocytes, which would allow a reduction in the number of animals used. Previous reports have shown that female age is an important factor when considering the efficiency of superovulation, but only one study was available that explored the effect of an ideal schedule for superovulation, using mature females and not younger females. The described range of ages being optimal for superovulation in B6 females included females that were less than 48 days old, at 21–32 days (Luo *et al.*, 2011; Kolbe *et al.*, 2015) or before vaginal opening at 24–30 days of age (Fox *et al.*, 2006; Caligioni, 2009). Despite weight also being described as an important factor, in our experiment no differences were found between the number of oocytes based on female weight or even when the weight variable was assumed as a weight class. The onset of puberty might be related to the efficiency of superovulation and some studies have stated that superovulation should be performed before onset of puberty, defined as vaginal opening at 24–30 days or 7 days later as described by other authors (Gaytan *et al.*, 2017). The relationship between the onset of puberty and weight in laboratory mice is not completely clear, as these two factors have been described as dependent or partially dependent (females with a higher body weight achieve puberty earlier) (Falconer, 1984, Yuan *et al.*, 2012). Conversely, recently investigations concluded that increase in body fat and leptin do not trigger the onset of puberty (Bronson, 2001). This study used females at ages that were already described as optimal for this kind of protocol. The fact that puberty may not be related to body weight can, in part, explain these results.

Despite age being described as an important factor, the time of administration has also an effect on the number of oocytes. This effect seems to be dependent on age, as 3-week-old females at 3 pm and 7 pm produced significantly better results compared with the other groups. When considering only the effect of schedule, regardless of female age, the 7 pm group had significantly higher numbers of oocytes compared with the 3 pm and the 5 pm (2 h before the dark cycle) groups. At 3 pm, the 3-week-old females produced significantly more oocytes compared with females at 4 and 5 weeks of age. The group with the better results was the 7 pm group, using females at 3 weeks of age and, while females at 3 weeks of age produced similar numbers of oocytes when superovulated at 3 or at 7 pm, older females gave better results when superovulated at 7 pm. For B6 females at 4 and 5 weeks of age, the onset of puberty was closer and synchronizing hormone administration with onset of the dark cycle appeared to produce better results. The fact that the strain tested responded better to superovulation protocols when hormones were given closer to the beginning of the dark cycle can be related to the fact that natural ovulation occurs 3–5 h after its start (Behringer *et al.*, 2016). As LH surge occurred about 6 h before ovulation (Hogan *et al.*, 1986) or 15–20 h after the midpoint of the dark cycle, this meant that, in a 7 am : 7 pm light cycle, LH surge occurred between 4 and 8 pm. hCG administration is recommended to be injected a few hours before the natural LH peak (Hogan *et al.*, 1986). However, for this particular strain and when using pre-pubertal females, synchronizing hCG injection with the natural LH surge seemed to produce better results for the three ages tested and, more significantly, for females at 4 and 5 weeks old compared with other schedules.

The estrous cycle phase at the time of hormone administration has been described by Tarín *et al.* (2002) as a factor with effects on the ovary response to superovulation. Here, synchronization levels in proestrus, estrus and metestrus were high at the time of hCG administration in all groups, but no significant differences were found between the mean number of oocytes from synchronized females. This finding reinforces the idea that the estrous cycle phase is not a determinant factor for superovulation outcome.

Our analysis excluded females with zero oocytes, as we considered that these females did not respond to superovulation; one female was in diestrus, and another three females were either in metestrus (one) or proestrus (two). This showed that synchronization in proestrus or metestrus is not enough to produce higher numbers of oocytes, and also that diestrus is the phase in which females produced the worst results.

Despite the larger numbers of oocytes collected in some of the discussed conditions, it has been described often that superovulation has a negative effect on embryo development (Van der Ertzeid and Storeng, 1992; Auwera and D'Hooghe, 2001; Fortier *et al.*, 2008; Market-Velker *et al.*, 2009). Other factors, such as age of females, has also been described as having a potential effect on the efficiency of IVF (Kolbe *et al.*, 2015). These last authors described lower IVF efficiency when oocytes were collected from females at 33–36 days of age compared with younger females. For this experiment, oocyte quality was not evaluated and further work would be required to validate a similar quality between groups.

In conclusion, for C57BL6/J mice, higher numbers of oocytes were produced when PMSG and hCG were given closer to the beginning of the dark cycle for females at 4 and 5 weeks of age. Females at 3 weeks of age produced better results at 7 pm and 3 pm, although the higher number of oocytes corresponded with females at 3 weeks at 7 pm. The percentage of females in proestrus in the 7 pm group was higher but, as already described, no significant differences were found between females in different estrous cycle phases. Females at 4 and 5 weeks of age responded better to superovulation when hormones are given closer to the beginning of the dark cycle (7 pm). Additionally, the use of females at 3 weeks of age at 3 and 7 pm could significantly reduce the number of females needed, especially compared with females at 5 weeks of age. Facilities can easily implement these findings by using light cycles that allow hormone administration over a schedule compatible with their working plan and by choosing females of optimal age for superovulation. This valuable and practical data can also help to reduce the number of euthanized female mice for collection of oocytes.

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Chapter 3

C57BL/6J and B6129F1 embryo transfer: unilateral and bilateral transfer, embryo number and recipient female background control for the optimization of embryo survival and litter size

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C57BL/6J and B6129F1 Embryo Transfer: Unilateral and Bilateral Transfer, Embryo Number and Recipient Female Background Control for the Optimization of Embryo Survival and Litter Size

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Simple Summary: Embryo transfer is a common procedure in rodent facilities related to rederivation protocols, recovery of cryopreserved embryos and production of genetically engineered animals. This procedure consists of the transfer of mouse embryos into the oviduct of a pseudopregnant recipient female in order to obtain live pups. The aim of this study is to further characterize the optimal conditions to perform embryo transfer using wild type strains and particularly the bilateral transfer. C57BL/6J and B6129F1 embryos were freshly collected and transferred to recipient females, after overnight culture to a 2-cell stage and tested for different conditions (unilateral and bilateral surgical procedures, variable number of embryos and reciprocity between recipient mother and embryo's genetic background). The results achieved show that C57BL/6J transfers with a low number of embryos provide higher success rates when using unilateral transfers, but for bilateral transfers a minimum number of embryos seems to be necessary. B6129F1 presented similar results, but bilateral transfers were more effective with low number of embryos. These results allow a better planning of the embryo transfer procedure, considering low number of embryos and the choice of unilateral transfers as the ideal condition for an optimal outcome. This optimization has a positive impact on the 3R's application: it can help to reduce the number of recipient and donor females and to improve recipient female's welfare through the use of a less invasive technique.

Abstract: Embryo transfer (ET) is a common procedure in rodent facilities. Optimizing this technique may help to reduce the number of animals, but little information is available regarding wild type strains and the conditions that affect embryo transfer. To explore this theme, 2-cell C57BL/6J embryos were transferred after overnight culture of freshly collected zygotes using different conditions: unilateral transfers using a total of 6, 8, 12, 15, 20 and 25 embryos were performed initially; then, this strain was also used for bilateral transfers using a total of 6, 12 and 20 embryos equally divided by the two oviducts. Groups of 25 embryos were not tested for the bilateral technique, since this condition produced the lower success rate when using the unilateral technique and 20 embryos would still represent a large number of embryos. A group of 2-cell B6129F1 embryos was also transferred using unilateral and bilateral ET with 6, 12 and 20 embryos. Crl:CD1(ICR) were used as recipient females for non-reciprocal transfers and C57BL/6J were used to test reciprocal transfers (only tested for six C57BL/6J unilateral transfers). Unilateral transfers using C57BL/6J mice produced higher success rates

using six embryos, compared to the other groups transferred unilaterally (p-values between 0.0001 and 0.0267), but the mean number of pups per litter was not different among groups. Bilateral transfer produced higher number of pups when 20 embryos were divided by the two oviducts compared to six (p = 0.0012) or 12 (p = 0.0148) embryos, but with no differences in success rates. No statistical differences were found between the groups of B6129F1, but better results were obtained on bilateral transfers using a total of six embryos. For the strain tested (C57BL/6J), the uterine environment (Crl:CD1(ICR) or C57BL/6J recipient) does not impact the outcome of the technique. These results complement previous work published using genetically engineered mice strains and show that unilateral transfers using low number of embryos (6), produce better outcomes when compared to bilateral or unilateral transfers using more embryos. It also highlights differences between the outcome of bilateral transfers in the two strains tested. A set of historical data of genetically engineered mice at a C57BL/6J background was also included, confirming that lower embryo numbers are related to higher success rates. Together, the outcome of these experiments can be important to reduce the number of recipient and donor females, optimize embryo transfers and improve animal welfare discouraging the use of a more invasive technique.

Keywords: embryo transfer; litter size; mouse unilateral and bilateral surgical transfers

1. Introduction

Embryo transfer (ET) is a key procedure related to assisted reproductive technologies (ART) in mice. The success of other techniques such as cryopreservation or in vitro fertilization (IVF), the efficiency of rederivation protocols and the production of genetically engineered mice all rely on the ability to produce a successful pregnancy after embryo transfer. Recently, several articles were published providing important insights about the factors that affect the ET efficiency, especially regarding the number of transferred embryos [1,2] and the surgical technique (bilateral or unilateral transfer) [3]. A reduced number of embryos transferred into the oviduct (between 8–12 embryos) [1] seems to produce a higher success rate measured as the ratio between the number of embryos transferred and the number of pups born. The work developed by Johnson et al. [4], using B6SJLF1 embryos, also concluded that transfers of 15 or more embryos did not result in more living fetuses.

Despite the variety of information available regarding ET, most of it is based on historical data from genetically engineered mice and it only considers a limited number of conditions: (1) data from genetically engineered mice is related to higher variability according to the different genetic modifications of each strain; (2) results obtained from specific number of embryos is lacking as the number of embryos analyzed is grouped in different intervals; (3) unilateral and bilateral transfers were only compared with each other when using the same number of embryos (15 to 18) and a wider group of conditions between unilateral and bilateral were not assessed; (4) bilateral transfers using small number of embryos were not evaluated; (5) genetic background reciprocity between the embryo and the receptive mother was also not explored. These gaps led us to test wild type strains in a larger group of experiments, testing a more complete and specific set of conditions whose results can be used as a basis for wild type strains ET. Although rederivation of mice is more commonly related to the transfer of genetically engineered mice between facilities or to produce a specific sanitary status within a facility, wild type strains are also frequently used for ET and little information is available about this subject in these strains.

Many factors can affect ET such as the pseudopregnancy state, the embryo quality and the genetic modifications, the background of the embryos or those related with the recipient female. Other factors such as the surgical technique (unilateral or bilateral) and the anesthetic and analgesic regimen selected are also relevant. Regarding pseudopregnancy, female receptivity to the male is higher at proestrus and visual observation of the vaginal mucosa [5] or synchronization of females through progesterone injection [6] are strategies to reduce the number of females needed to obtain

pseudopregnant females. Embryo implantation after ET was described to be more efficient in females at diestrus when blastocyst transfer is performed [7]. Uterine receptivity and implantation rates are also strongly dependent on the embryo and endometrium interaction [8,9], since blastocysts regulate key molecules for the implantation process. Another factor for a successful ET is the embryo quality, which can be compromised either by in vitro maturation [10] or by superovulation. Superovulation, commonly used as a first step to obtain embryos for ET, has been described as having an evident impact on embryo development [11–15] and can affect the final ET result. The embryo genetic background [2,16,17] and genetic modifications [18] may also impact the implantation rate and the number of pups born. Finally, the surgical technique should also be considered when performing ET. The surgical technique selected may present variations regarding the anatomic location of the transfer (depending on the embryo developmental phase), although most commonly involving the oviduct. At this location, a variable number of embryos can be transferred unilaterally or bilaterally. The later demands a more invasive surgical approach, but with potential to produce higher number of living fetuses when compared to unilateral transfers [3]. Pregnancy rates are described as being higher when small number of embryos (<21) are used [2]. Furthermore, the ability to produce a pregnancy after ET has been reported as possible to be achieved with less than five embryos [2], 8 to 12 embryos [1] or less than 15 embryos [4] through unilateral transfer. Additionally, the analgesic and anesthetic protocol must be considered as a potential source of variation although several options are described as having no significant effect on the ET outcome [19–23], such as carprofen, used for this experimental work.

The aim of this experiment was to compare a different number of embryos from two defined mouse genetic backgrounds (C57BL/6J and B6129F1), transferred unilaterally and bilaterally and also to compare the most efficient condition using reciprocal and non-reciprocal embryo transfer methods. We started by analyzing all ET from genetically engineered mice at a C57BL/6J background performed in our facility during the last 2.5 years. Then, unilateral transfers in C57BL/6J using 6, 8, 12, 15, 20 and 25 embryos were tested. The experiment was then repeated using bilateral transfers with a total of 6, 12 and 20 embryos, equally divided by both oviducts. Unilateral and bilateral ET was then repeated by transferring 6, 12 and 20 embryos using a different wild type strain donor (B6129F1). The main goal was to understand the impact of both the embryo's number and the surgical technique used for ET in wild type strains and to complement the data available using genetically engineered mice historical data, especially regarding bilateral transfers using small number of embryos. These results may contribute both to improve animal welfare and reduce the number of females involved in this process. It will also allow a practical optimization of ET protocols in rodent animal facilities.

2. Materials and Methods

The experimental work was approved by the i3S Animal Welfare and Ethics Review Body (AWERB) and submitted for approval at the Portuguese Competent Authority (DGAV), (project reference 2017_03). Experiments were performed ensuring animal welfare and according to the European legislation (Directive 63/2010).

2.1. Animals

Animals were born and kept at the i3S animal facility and were maintained in regular conditions: temperature was maintained between 20 °C and 24 °C and humidity between 45% and 65%; distillated water was provided ad libitum as well as autoclaved feed (2014S Envigo diets); a corn cob bedding was used and environmental enrichment (paper tube roles) and paper for nesting were provided inside the cages. The Crl:CD1(ICR), C57BL/6J and 129/SvPasCrl colonies were originally bought to Charles River; breeders are replaced frequently in order to maintain the genetic integrity of the strains. Females were housed in 1264C Eurostandard Type II (groups of up to 5 females) or 1290D Eurostandard Type III (groups of 6 to 10 females) cages; males were housed in 1264C Eurostandard Type II cages (1 animal per cage for the breeding). C57BL/6J and 129/SvPasCrl were free from the following agents: MHV, EDIM, MPV, MVM, PVM, Sendai virus, TMEV, Ectromelia, LCMV, MAD-1 and 2, Mouse *Cytomegalovirus*,

Reovirus, *Bordetella bronquiseptica*, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutsheri*, *Cryptosporidium* spp., *Mycoplasma pulmonis*, *Pasteurella* spp., *Pseudomonas aeruginosa*, *Salmonella* spp., *Helicobacter* spp., *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptobacillus moniliformis*, *Klebsiella pneumoniae*, ectoparasites (*Myobia, Radfordia* and *Myocoptes* spp.), *Eimeria* spp., *Entamoeba muris*, *Giardia* spp., *Spironucleus muris*, *Tritrichomonas muris*, *Aspiculuris tetraptera* and *Syphacea obvelata* and *muris*. Occasional positives were found at quarantine (origin of animals from the genetically engineered mice at a C57BL/6J background) for *Pasteurella* spp., *Helicobacter* spp., *Klebsiella pneumoniae* and *Staphylococcus aureus* as animal origin differs among the two experiments.

2.2. Experimental Groups

This work includes 3 sets of experiments (summarized in Figure 1) one set involving historical data of genetically engineered mice at a C57BL/6J background, using unilateral and bilateral non-reciprocal embryo transfers; a second set of prospective experiments involving reciprocal and non-reciprocal unilateral and bilateral transfers with C57BL/6J wild-type mice; a third group of prospective experiments involving non-reciprocal unilateral and bilateral transfers with B6129F1 wild-type mice. The choice for the number of embryos tested in the bilateral condition was based on the results of the unilateral transfer.

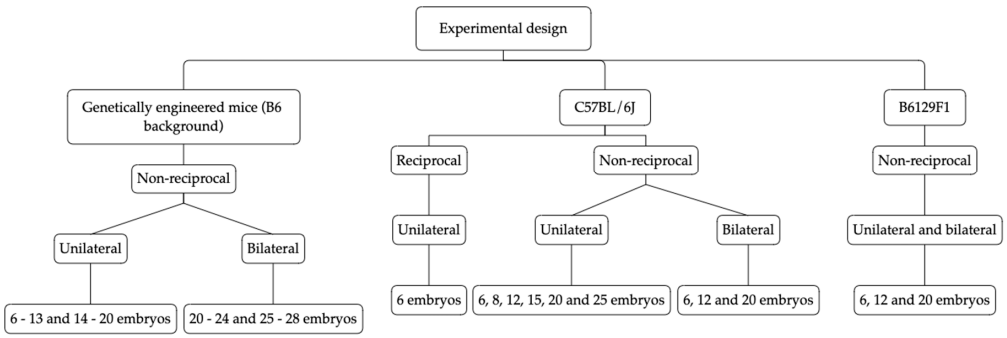

Figure 1. Experimental design and groups.

Genetically engineered mice at a C57BL/6J background historical data: a total of 39 sessions of ET performed unilaterally and 35 sessions of bilateral ET from a total of 36 genetically engineered mice strains at a C57BL/6J background were analyzed, using non-reciprocal transfers. The number of 2-cell embryos transferred for the unilateral technique was between 6 and 22 and for the bilateral transfer was between 15 and 28. For easier comparison, unilateral transfers were grouped in 6–13 and 14–22 transfers of 2-cell embryos. Bilateral transfers were grouped in transfers using 20–24 and 25–28 embryos.

Data from C57BL/6J mice: a controlled experiment using wild-type mice (C57BL/6J) was done. For this experiment embryo transfers were performed according to the following conditions: non-reciprocal embryo transfer was performed using C57BL/6J 2-cell embryos transferred in groups of 6, 8, 12, 15, 20 and 25 unilaterally or 6, 12 and 20 embryos transferred bilaterally into CD1 pseudopregnant females (equally by the two oviducts); reciprocal embryo transfers were performed using 6 C57BL/6J embryos, transferred unilaterally to C57BL/6J pseudopregnant females. Transfers were performed during the morning and each female was allocated to a group in a random way.

Data from B6129F1 mice: another set of experiments was performed using B6129F1 embryos. Unilateral and bilateral non-reciprocal transfers using 6, 12 and 20 embryos were performed and CD1 pseudopregnant females were used as recipients. Transfers were also performed during the morning and each female was allocated to a group in a random way.

2.3. Superovulation

PMSG (ProSpec, HOR 272) and hCG (ProSpec, HOR 250) were diluted at a dose of 50 IU in sterile water and frozen at −20 °C until further use. Before injection, aliquots were thawed and diluted in sterile water to get a 100 µL dosage of 5 IU for intraperitoneal injection.

PMSG and hCG were given at an interval of 48 h. Hormones were given at 3:00 p.m. at a light cycle of 12:12-h light:dark cycle, lights on at 7:00 a.m. At the time of hCG injection, females were mated with males overnight and checked for plug in the following morning.

For the genetically modified mice at a C57BL/6J background historical data, males used were between 4 and 6 months of age, with proved fertility and donor females between 3 and 5 weeks of age.

For the C57BL/6J experiment, males were between 4 and 6 months of age, with proved fertility; males were used once per week allowing a one week resting period between matings. C57BL/6J donor females were between 3 and 5 weeks of age.

The B6129F1 experiment used 129/SvPasCrl males between 3 and 5 months of age and a similar breeding scheme as the one described for the C57BL/6J males. These males were crossed with C57BL/6J donor females between 3 and 5 weeks of age.

2.4. Embryo Collection

To collect embryos at 0.5 dpc, plug positive females were euthanized in the morning after the mating by cervical displacement. Oviducts were collected in M2 media [24] and, after tearing the ampulla, the cumulus cells surrounding the zygotes were digested in M2 supplemented with 0.5-mg/mL hyaluronidase (Sigma-Aldrich, H4272) for less than 1 min. After digestion, embryos were washed in 50 µL M2 drops, followed by several washes in 50 µL KSOM drops (EmbryoMax® KSOM Mouse Embryo Media, ref. MR-020P-5 F) and incubated in the same media drops using the same volume covered with mineral oil (Mineral oil, Sigma-Aldrich, ref. M8410). In the following morning, embryos at 1.5 dpc were washed in M2 and transferred to a pseudopregnant Crl:CD1(ICR) female or C57BL/6J (in case of testing reciprocity).

2.5. Embryo Transfer

Genetically engineered mice at a C57BL/6J background historical data: Crl:CD1(ICR) females were used as recipient females for the embryos (non-reciprocal). Before mating, CD1 female mice were housed in 1290D Eurostandard Type III cages in groups of 8 to 10 females. For the embryo transfer, 0.5 dpc pseudo-pregnant females (age between 5 to 10 weeks) were obtained by crossing surgically vasectomized CD1 males with demonstrated infertility (8 to 16 weeks of age) with CD1 females. Surgical anesthesia was obtained with a mixture of intraperitoneal ketamine (80 mg/kg, Clorketam) and medetomidine (1 mg/kg, Sededorm); carprofen (0.5 mg/kg, Rymadil) was administered subcutaneously before the surgery and kept during the first 48 h after surgery. A heating pad, eye ointment (Siccafluid, Unidoses 2.5 mg/g, Laboratoires THÉA) and aseptic technique were applied before surgery. The surgical procedure was started when the withdrawal reflex was not present. Atipamezole was used for female's recovery (1 mg/kg, Revertor). For this group, only unilateral transfers were performed, always on the left oviduct. Briefly, a 0.5 to 1 cm incision on the left dorsal side of the abdominal wall followed by an incision on the peritoneal wall were performed in order to expose the ovary fat. Using blunt forceps, the ovarian fat was removed from the peritoneal cavity to expose the oviduct and ovary. Bulldog forceps were then applied to the ovarian fat and the oviduct was identified under the stereomicroscope. Only females with a large ampullae were used for the embryo transfer. Once the ampullae was identified, embryos were placed inside a pulled glass capillary with minimum M2 media, followed by a small air bubble. A small cut using spring scissors was performed in the area before the ampullae, after the infundibulum, just large enough to introduce the capillary. After capillary insertion, the embryos were placed inside the oviduct and the ovary and oviduct were returned to the abdominal cavity. The peritoneum and the skin were sutured using polyglycolic acid, 6-0 suture line.

Data from C57BL/6J and B6129F1 wild-type mice: For these experiments' recipient females (Crl:CD1(ICR) for the non-reciprocal and C57BL/6J for the reciprocal groups) were anesthetized using isoflurane (5% for induction and 2%–3% for maintenance with oxygen at 1L/min for induction and 0.2 to 0.3 L/min for maintenance). Carprofen was also used for analgesia and the same surgical procedure

described above for unilateral transfers of genetically engineered mice was used. For the unilateral transfers using C57BL/6J as recipient females (reciprocal), the anesthesia, analgesia and surgical procedure were the same as described for unilateral transfers of wild type C57BL/6J and B6129F1 mice. C57BL/6J recipient females were used between 10 and 16 weeks of age and mated with surgically vasectomized CD1 (8 to 16 weeks) males with demonstrated infertility to induce pseudopregnancy.

Bilateral transfers were performed using the same anesthetic and analgesic protocol described for the genetically engineered mice. The surgical procedure consisted in doing an incision over the lumbar area (0.5 to 1 cm) and exposition of the peritoneal wall on both sides. A second incision was performed at the peritoneum wall from the left side and a similar procedure to the described for the unilateral transfer was done for the embryo transfer. The procedure was repeated on the right side and, after closing both peritoneum incisions, the skin was sutured using polyglycolic acid, 6-0 line. Bilateral transfers were all performed using non-reciprocal transfers with a total of 6, 12 and 20 embryos in C57BL/6J and B6129F1, equally divided by the two oviducts.

2.6. Statistical Analysis

GraphPad Prism version 8.00 for Mac, GraphPad Software, La Jolla, CA, USA, www.graphpad.com was used for statistical analysis. We assessed data for normality using the D'Agostino–Pearson normality test whenever the number of observations allowed its application; alternatively, the Kolmogorov–Smirnov test was used when the sample did not allow the use of the first test. We used a Mann–Whitney test to compare the historical data. In this group only the number of pups born was compared as two independent groups considering unilateral and bilateral transfers, as the variation between the mean number of embryos used for unilateral and bilateral transfer was high and success rates and the number of pups could not be compared. Success rates were only compared using the same test for the two unilateral groups and a second independent test was performed for the two bilateral groups. For the C57BL/6J experiment, a one-way ANOVA followed by a Tukey's multiple comparison test was done between the unilateral transfers and a second set of comparisons was used for the bilateral group. Reciprocal and non-reciprocal transfers using 6 embryos were equally analyzed using a Student's *t*-test. Transfers using the same number of embryos transferred bilaterally and unilaterally were subsequently analyzed using a two-way ANOVA, as well as the B6129F1 transfers. The comparison between the C57BL/6J and B6129F1 transfers was performed using a three-way ANOVA. A *p*-value of 0.05 or less was considered as a statistically significative result.

3. Results

Genetically engineered mice at a C57BL/6J background historical data: we aimed to compare ET using different number of embryos for unilateral and bilateral transfers using historical data from genetically modified mice. Since the mean number of embryos transferred was distinct between unilateral and bilateral methods, results were grouped according to the number of embryos used. For unilateral and bilateral transfers, two groups were established as stated in Table 1. We observed that the mean number of pups born after unilateral transfers using 6 to 13 embryos was lower comparing to unilateral transfers using 14 to 22 embryos ($p = 0.0196$). No statistical differences were found between the two groups for bilateral transfers ($p = 0.3292$). Success rates, defined as the percentage of embryos that gives origin to a live pup, between the two unilateral transfer groups and between the bilateral transfer groups were not significantly different ($p = 0.1865$ and $p = 0.0724$, respectively). Pregnancy rates were also not significantly different between the 4 groups ($p = 0.159$).

Data from C57BL/6J: We aim to further understand the impact of both the embryo's number and the surgical technique used for ET in a wild type strain (C57BL6/J), especially regarding bilateral transfers using small number of embryos. Mean number of pups born and the success rates are shown in Table 2. As for non-reciprocal unilateral transfers, the mean number of pups born was not statistically different between the different unilateral groups, meaning that the size of the litter does not increase significantly with an increase in the number of embryos transferred (*p*-values between 0.1079

and 0.999). On the other hand, when comparing the success rate of unilateral transfers, statistically significant differences were found between transfers of 6 and 15 embryos (77.8% vs. 42.7%, $p = 0.0062$), between 6 and 20 (77.8% vs. 40%, $p = 0.0029$), between 6 and 25 (77.8% vs. 24%, $p < 0.0001$), between 6 and 12 (77.8% vs. 45%, $p = 0.0117$) and between 8 and 25 embryos (55% vs. 24%, $p = 0.0267$). For the non-reciprocal bilateral transfers significant statistical differences on the mean number of pups born were found between 6 and 20 embryos ($p = 0.0012$) and between 12 and 20 embryos ($p = 0.0148$); the mean number of pups was not statistically different between 6 and 12 embryos ($p = 0.5821$). Regarding the success rate, no significant differences were found between any of the non-reciprocal bilateral groups (p-values between 0.5083 and 0.9486). Unilateral and bilateral non-reciprocal embryo transfers using the same number of embryos were also compared: a statistically significant increase in the success rate was found when comparing 6 embryos unilateral transfer (77.8%) to: 6 embryos bilateral transfer (35.7%, $p = 0.0065$), 12 embryos bilateral transfer (31.9%, $p = 0.0038$), 20 embryos bilateral transfer (45.7%, $p = 0.0038$) and 20 embryos unilateral (40%, $p = 0.0405$). The number of pups was significantly different between 6 embryos bilateral group and 20 embryos unilateral ($p = 0.0166$) and bilateral ($p = 0.0008$) groups and between 12 and 20 embryos bilateral groups ($p = 0.0257$). Then, we tested reciprocity to assess whether the genetic background of recipient females was associated with an increased success rate. No statistical differences were detected between 6 C57BL/6J embryos transferred into a CD1 or C57BL/6J pseudopregnant female ($p = 0.1828$). Figure 2 represents the results for the C57BL/6J mice using 6, 12 and 20 embryos unilateral and bilateral transfers. The mean number of embryos needed to obtain one live pup are represented in Table 3.

Table 1. Genetically engineered mice at a C57BL/6J background transfers, grouped by number of embryos.

Reciprocity	Surgical Technique	Number of Transfers	Total Number of Embryos Transferred	Pregnancy Rate (%)	Mean Number of Pups Born per Litter [a]
Non-reciprocal	Unilateral	17	6 to 13	100	5.5 ± 0.4
		22	14 to 22	77.3	7.1 ± 0.4
	Bilateral	23	20 to 24	73.9	6.4 ± 0.4
		12	25 to 28	83.3	5.5 ± 0.6

Total number of transfers, number of embryos transferred, pregnancy rates and mean number of pups born after unilateral and bilateral transfers using genetically engineered mice at a C57BL/6J background. For bilateral transfers, embryos were divided equally by the two oviducts. [a] Values are mean ± SEM.

Table 2. C57BL/6J transfer groups and results.

Reciprocity	Surgical Technique	Total n of Embryos Transferred	Number of Transfers	Number of Pregnant Females	Pregnancy Rate (%)	Number of Pups Born per Litter [a]	Success Rate (%) [a]
Non-reciprocal	Unilateral	6	7	6	85.7	4.6 ± 0.3	77.8 ± 5.6
		8	7	5	71.4	4.4 ± 0.6	55 ± 7.5
		12	7	5	71.4	5.4 ± 1.08	45 ± 9
		15	7	5	71.4	6.4 ± 0.4	42.7 ± 2.7
		20	6	5	83.3	8 ± 1.6	40 ± 7.9
		25	6	5	83.3	6 ± 0.9	24 ± 3.6
	Bilateral	6	7	7	100	2.1 ± 0.6	35.7 ± 9.2
		12	6	6	100	3.8 ± 0.9	31.9 ± 7.3
		20	7	7	100	9.1 ± 1.7	45.7 ± 8.4
Reciprocal	Unilateral	6	7	6	85.7	3.8 ± 0.5	63.9 ± 8

Number of transfers, pregnant females, mean number of pups and success rate (number of pups born/number of embryos transferred) for C57BL/6J are summarized at table for each of the experimental conditions (reciprocal and non-reciprocal; unilateral and bilateral and according to the number of embryos transferred). [a] Values are mean ± SEM.

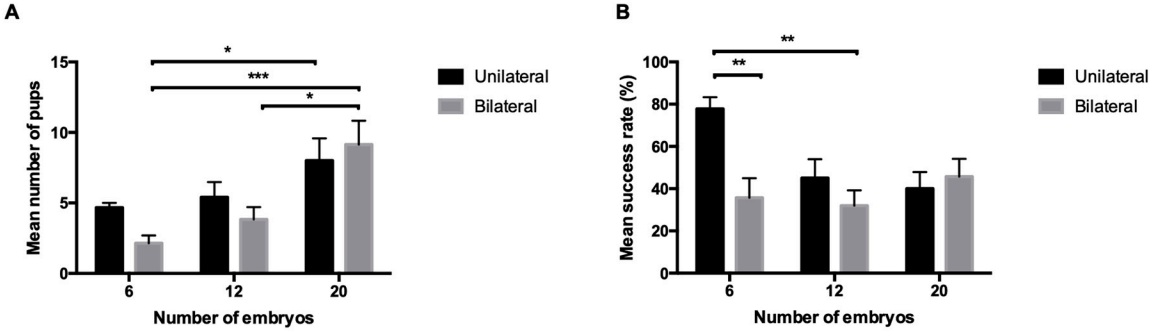

Figure 2. (**A**) Mean number of pups after unilateral and bilateral transfers using 6, 12 and 20 C57BL/6J embryos. Statistically significant differences were found between 6 embryos unilateral group and 6 and 12 embryos bilateral; (**B**) success rates for unilateral and bilateral transfers using 6, 12 and 20 embryos. Statistically significant differences were found between 6 embryos bilateral and 20 embryos unilateral and bilateral and between 12 and 20 embryos bilateral. Values are mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3. Embryo effort for the generation of one live pup using C57BL/6J and B6129F1.

Reciprocity	Surgical Technique	Total Number of Embryo's Transferred	C57BL/6J [a]	B6129F1 [a]
Non-reciprocal	Unilateral	6	1.3 ± 0.1	3.1 ± 0.9
		8	1.8 ± 0.5	–
		12	2.8 ± 0.8	1.6 ± 0.2
		15	2.4 ± 0.17	–
		20	3 ± 0.7	3.7 ± 1.3
		25	4.5 ± 0.6	–
	Bilateral	6	3.8 ± 0.8	1.6 ± 0.4
		12	4.7 ± 1.6	2.1 ± 0.3
		20	3.3 ± 1.2	4.6 ± 1.5
Reciprocal	Unilateral	6	1.7 ± 0.3	–

Embryo effort measured as the number of embryos needed to obtain one live pup using C57BL/6J and B6129F1. [a] Values are mean ± SEM.

Data from B6129F1: We aim at evaluating the mean number of pups and the success rates in a second hybrid strain. Only non-reciprocal transfers were performed for this strain and no statistical differences were found between the success rates (p-values between 0.2317 and 0.9999) or between the mean number of pups (p-values between 0.1231 and 0.9999) (Table 4, Figure 3). The mean number of embryos needed to obtain one live pup are represented in Table 3.

Table 4. B6129F1 transfer groups and results.

Reciprocity	Surgical Technique	Total n of Embryos Transferred	Number of Transfers	Number of Pregnant Females	Pregnancy Rates (%)	Number of Pups Born per Litter [a]	Success Rate (%) [a]
Non-reciprocal	Unilateral	6	7	6	85.7	3 ± 0.8	50 ± 12.9
		12	7	6	85.7	7.8 ± 0.9	65.3 ± 7.2
		20	7	6	85.7	7 ± 2.2	36.7 ± 6
	Bilateral	6	7	5	71.4	4.4 ± 0.7	73.3 ± 11.3
		12	7	6	85.7	6.7 ± 1.2	55.6 ± 10.2
		20	6	5	83.3	7.3 ± 2.2	35 ± 11.6

Number of transfers, pregnant females, mean number of pups and success rate (number of pups born/number of embryos transferred) for B6129F1 strain are summarized at table for each of the experimental conditions (non-reciprocal; unilateral and bilateral and according to the number of embryos transferred). [a] Values are mean ± SEM.

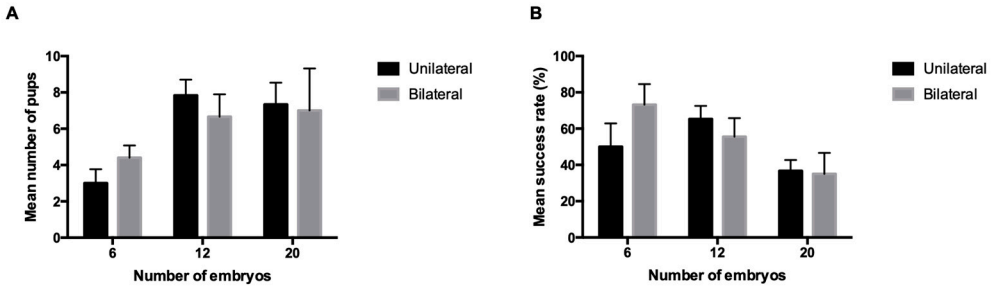

Figure 3. (**A**) Mean number of pups after unilateral and bilateral transfer for B6129F1 transfer groups (6, 12 and 20 embryos); (**B**) success rates for unilateral and bilateral transfer for B6129F1 transfer groups (6, 12 and 20 embryos). Values are mean ± SEM. No statistically significant differences were found between the groups.

Comparison between C57BL/6J and B6129F1 results: we aim at comparing the unilateral and bilateral ET techniques using 6, 12 and 20 embryos in the two different strains. The comparison of these conditions between C57BL/6J and B6129F1 strains, revealed no significant differences between the two strains for the success rates, but significant differences were found for the number of pups in transfers using 6 C57BL/6J embryos bilateral and 12 B6129F1 embryos unilateral ($p = 0.0325$) and between 6 B6129F1 embryos unilateral and 20 C57BL/6J bilateral ($p = 0.0144$).

4. Discussion

This study presented the differences in the outcome of ET using two wild type strains, with a particular interest in bilateral transfers, that were until now only characterized for 15 to 18 embryos and using genetically engineered mouse strains [2]. This work is also the first to characterize ET in wild type strains as, to date, all the available data were focused on historical data obtained from genetically engineered mice. A set of historical data corresponding to genetically engineered mice at a C57BL/6J background was also included. These data are not directly comparable to the prospective study using C57BL/6J and B6129F1, but can, in part, be compared to the already available data on this topic. Our results show, for the genetically engineered mice at a C57BL/6J background, that lower number of pups are obtained when using unilateral non-reciprocal transfers with 6 to 13 2-cell embryos compared 14 to 22 embryos ($p = 0.0196$), but the success rates are not significantly different. This result is in accordance with the findings of Gonzalez-Jara et al. [1], who concluded that smaller number of embryos transferred unilaterally are related to a higher success rate in genetically engineered mice. Despite both data correspond to C57BL/6J embryos, our results revealed a mean number of pups per litter higher than the previously mentioned author. Both studies used CD-1 as receptive females, but the anesthesia and analgesia were distinct (isoflurane versus ketamine and medetomidine); the embryo collection method was also different (our investigation involves incubation). Nevertheless, the simple fact that the animal's genetic alterations were distinct may explain the small differences obtained. The anesthesia and analgesia regimen involved in both studies has been described as having no significant effects on embryo implantation [23,25]. However, superovulation and incubation may affect in vitro and in vivo embryo development [13], and the in vitro culture seems to have a lower impact when compared to a stimulated uterine environment after PMSG/hCG administration [14,26], which can, in part explain the higher mean number of pups herein obtained.

The prospective study using C57BL/6J revealed no differences between the groups, when comparing the mean number of pups in unilateral transfers. However, the success rates for the unilateral transfer using C57BL/6J were significantly different between those using 6 embryos unilaterally with all the other groups. These results corroborate previously published data showing that a low number of embryos has a better outcome than a higher number [1]. The embryo effort, measured as the number of embryos needed to obtain one live pup, is lower when using smaller number of embryos for the transfer: the unilateral transfer using 6 embryos needs a mean number of 1.3 embryos to produce one live pup.

Notwithstanding, this number is substantially increased when using 12 (2.8), 15 (2.4) or 20 (3) embryos. Despite being able to produce a normal pregnancy rate, the 25 embryos group, resulted in the lowest success rate and the higher embryo effort to produce one live pup (4.5). Obtaining better results in conditions that involve a lower number of embryos may be related to the uterine capacity and ability to have free space for implantation, as described by Johnson et al. in 1996 [4]. This author used a different strain and only evaluated the number of implantation sites and reabsorptions, without a full pregnancy, but described 11–12 fetuses as the maximum uterine capacity. A slightly higher value (15) was found in our work (a litter of 15 pups obtained after a 20 unilateral transfer using C57BL/6J). The result for the group involving 15 to 25 embryos may also be related to the fact that mice have a duplex uterus that includes a divided cervix, that avoids embryo's uterine migration [27]. This characteristic may be the cause for the lower success rates related to ET using high number of embryos, as the uterine capacity is exceeded. Overall, the mean number of pups obtained in the different groups is higher when compared to the work published using unilateral transfers from Gonzalez-Jara et al. and Dorsch et al. [1,2], except for transfers using 25 embryos (7.5 pups described by Gonzalez-Jara et al. versus 6 pups obtained in our prospective work). These differences related to higher mean number of pups, may be related to the fact that the prospective study presented here used wild type strains, with a lower variability and possibly higher embryo capacity to survive after ET. In addition, other results available in the literature grouped the embryos in different classes, impairing proper comparisons. The same can be said regarding the success rates, our work using C57BL/6J wild type animals is related to slightly higher pregnancy rates when compared to the work of Gonzalez-Jara et al.

Bilateral transfers are most commonly used when a large number of embryos is intended to be transferred, but little information was available regarding lower number of embryos. The prospective study using C57BL/6J revealed significant differences between the mean number of pups using 6 and 12 embryos and between 12 and 20 embryos, with higher mean numbers of pups being related to the 20 embryos group transfer. For this strain, better success rates were also related to higher number of embryos, a contradictory result when considering the numbers obtained for unilateral transfers. Groups of 3 to 5 embryos were already described as enough to produce pregnancy [2] but this effect was, until now, only evaluated for unilateral transfers.

Using a different strain (B6129F1), a F1 hybrid, no relevant differences between the conditions tested for the unilateral transfer were detected, revealing again that lower embryo numbers are able to produce more efficient outcomes. Bilateral transfers with B6129F1 also did not produce significant differences, but, in contrast to the results obtained with C57BL/6J for bilateral transfers, B6129F1 displayed a higher success rate when lower number of embryos were used. This effect on bilateral transfers, although not statistically significant, may suggest that there are strain dependent differences that, perhaps with a larger set of animals could be better understood. The size of B6129F1 pups may explain the inability to have better outcomes when using a larger number of pups [28]. C57BL/6J, on the other hand, are smaller and the better results obtained with 20 embryos bilateral transfers may be related to this strain characteristic. It is interesting to notice that 6 embryos using the bilateral technique produced two times more pups in B6129F1, when compared to C57BL/6J. The genetic background may, again, explain the differences found as both the mother and embryos background may affect the embryo development [29]. Mouse embryo implantation demands a fine tune between the embryo and the mother uterine cavity, with the balance between estrogens and progesterone being essential for implantation to occur [30–32]. There is also evidence that embryo manipulation, such as embryo culture and the use of superovulation have detrimental effects on embryo survival [14,33–37], as already stated. These effects may have distinct levels when different strains are considered, such as different strains survive differently to other type of manipulations, such as microinjection [38].

Regarding the background of the recipient female, no differences were found when embryos were transferred into an oviduct of the same background (B6) or into a different background (CD1). Considering that CD1 females tend to have good maternal skills, it is more efficient to use a non-reciprocal transfer in this case. This may be questionable if other backgrounds or genetic

modifications are considered It is known that the survival of embryos is more dependent on the mother background than on the embryo background [39] and other authors also described differences between C3H, NMRI and DBA mice as recipient females [16].

These results can significantly impact the number of embryo donor's and maximize the collected embryo's use. For the C57BL/6J, six embryos transferred unilaterally produced the most efficient success rate (77.8%) with expected mean numbers of 4.6 pups. B6129F1 had the best outcome when six embryos were transferred bilaterally (mean number of pups of 4.4 and a success rate of 73.3%). Although no significant differences were found, B6129F1 seems to have better results when 20 embryos are used bilaterally, when compared to the same condition in C57BL/6J. Recipient females used can equally be optimized by planning the number of needed transfers according to the expected size of litters described. Bilateral transfers do not seem to offer any advantage from a success rate point of view and require a more complex, time consuming and invasive option. Thus, the choice for a unilateral transfer improves animal welfare. In the particular case of the B6129F1 strain, the bilateral transfer when having low number of embryos (six embryos) seems to offer some advantages although these differences were not significative.

5. Conclusions

In conclusion, for wild type C57BL/6J, better results after embryo transfer were obtained when using six embryos unilaterally: success rates of unilateral transfers decrease when the number of embryos increases. The mean number of pups does not increase proportionally to the number of embryos transferred when using unilateral transfers, but, in bilateral transfers, higher mean numbers of pups are obtained when 20 embryos are introduced. Thus, when using bilateral transfers in C57BL/6J, more than 12 embryos should be considered as a total for the transfer. B6129F1 behave similarly, with better outcomes when using lower number of embryos unilaterally. Nevertheless, bilateral transfers in this strain had better results with a lower number of embryos, perhaps due to the size of B6129F1 pups. Bilateral transfer does not seem to offer any obvious advantage and represents a more invasive technique with a probable higher negative impact on animal welfare. To date, no information was available regarding ET of wild type strains, which may serve as a more reliable basis for ET planning. An effort to reduce the number of animals used must be done by optimizing common techniques such as ET. Together with other information related to the best practices when using superovulation and incubation, these results can help the entire process optimization and significantly reduce the number of donor females needed for ET, helping to improve the efficiency of ET by increasing the number of live pups obtained after transfer.

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Chapter 4

The impact of the gut microbiota in the phenotypic characterization of an intestinal tumorigenesis mouse model raised in different areas of the animal facility

Article in preparation

The impact of the gut microbiota in the phenotypic characterization of an intestinal tumorigenesis mouse model raised in different areas of the animal facility

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Article in preparation

Abstract

Microbiota variability has the potential to change experimental results and impairs reproducibility. We aim to characterize the gut microbiota content and phenotype of VCMSH2$^{LoxP/LoxP}$ mice, a spontaneous model of cancer in the small intestine maintained in two different areas of the animal facility (barrier and experimental zones). The gut microbiota, tumor burden, inflammatory scores, $CD8^+$, $CD4^+$ T cells and lactoferrin levels were compared at 10 months of age. Differences in alpha diversity (evenness) ($P = 0.0008$) and in the relative abundance of different phyla with inverted dominances of common phyla such as Bacteroidetes and Firmicutes were found. Positive and negative correlations between inflammatory scores and a few microorganisms were found in the barrier. Lactoferrin levels at 10 months were also negatively correlated with the presence of *Enterorhabdus* sp. and *Fusicatenibacter* sp. (both $P = 0.03$) in the barrier. $CD8^+$ T cells were higher at the barrier zone but lactoferrin levels were increased in the experimental group. These values were, however, not significantly different when the presence of tumor was considered. This study emphasizes the importance of microbiota characterization as a tool to reduce variability and increase experimental reproducibility.

Introduction

The gut microbiota represents the different microorganisms that inhabit the gastrointestinal tract, including bacteria, viruses, archaea, protozoa, fungi and yeasts. In the recent years, the microbiota impact on experimental results has become a relevant element of study and variable in many experimental settings. This topic is of central importance, since it is now known that microbiota contributes to the development of immune mediated diseases such as type 1 diabetes, inflammatory bowel disease, obesity, and asthma, and to various types of cancer, including colon cancer [253, 278-282].

Variability in gut microbiota of different strains [234] and between individuals of the same strain but with distinct origins [234, 283] has been described, as well as in animals of the same animal facility, housed in different rooms [206]. The effect that different animal

suppliers may have on the microbiota can even overcome the effect of different diets [284]. This variability impairs a thorough comparison of results, reproducibility and has a negative impact on the number of animals needed for a specific experiment. Although standardization between animal facilities may be difficult to achieve, some good practices should be implemented for a better understanding of scientific results [285]. Moreover, an effort to characterize the possible impact that the microbiota may have in specific models of disease should be considered.

The characterization of the microbiota in cancer animal models is particularly important as the immune system, the microbiota, and cancer development are intrinsically connected.

The development of the immune system and the response to different challenges is shaped and dependent on the microbiota. Early colonization of the gut is essential to direct the immune system towards an immunotolerant state [247], characterized by low levels of TLR activity in the first weeks of life. This colonization starts before birth, as revealed by the unique microbiota found in the placenta [286] and continues with the contact with the vaginal microbiota [287]. Germ-free animals enhanced the central role of the gut microbiota in the immune system and health: these animals are characterized by low IgA levels, underdeveloped and fewer intestinal Peyer patches and reduced levels of several cellular types such as $CD4^+$ and $CD8^+$ T cells [254].

Some microorganisms, such as *Bacteroides fragilis*, are essential to maintain the gut homeostasis by promoting the differentiation of Tregs [288] and segmented filamentous bacteria (SFB) are described as being essential in mice to the differentiation of Th17 cells [289]. Butyrate, a short-chain fatty acids (SCFA) produced by bacteria is used as an important source of energy for colonocytes [290]. Tumoral cells are unable to use it as a source of energy and it has been described for its antitumoral effects via inhibition of histone deacetylase (HDAC) [291].

Accordingly, the microbiota can affect tumor development and its actions can be contradictory, particularly in the dual role of promoting or inhibiting tumor progression. Dysbiosis can lead to the overgrowth of pathogenic bacteria and consequent cell injury through direct DNA damage as it is the case of *E. coli* [292], or may promote cell proliferation through β-catenin pathway activation in the case of *Fusobacterium* [293]. In fact, colorectal cancer patients have a distinct and less diverse gut microbiota in comparison with healthy controls [294]. Other microorganisms are recognized for their potential to improve cancer therapy and patient prognosis: it is the example of immune checkpoint blockers inhibitors (mainly CTLA-4 and PD-1 inhibitors) [295] and cyclophosphamide action dependency on *Lactobacillus johonsonii* [269].

Several animal models of cancer related to the digestive system are used to better understand disease mechanisms or to test for new drugs. Lack of reproducibility of animal

experiments has been a discussion theme in the last years and the highly variable gut microbiota certainly contributes to this problem [296].

The aim of this work consisted in comparing the gut microbiota of the mouse model VCMSH2$^{LoxP/LoxP}$ between the barrier and experimental areas in order to evaluate the impact of the microbiota in this model phenotype. To do it, the inflammatory pattern, tumor burden, lactoferrin and the gut microbiota were evaluated in two distinct zones of the facility (barrier and experimental zones). Animals were followed until 10 months of age. Differences in alpha diversity, beta diversity and dominant phyla were found as well as differences on inflammatory scores, CD8$^+$ T cells and lactoferrin levels.

Materials and methods

Animals – housing and sample collection

All the experiments were performed at the i3S Animal Facility, under a project licensed by the Portuguese Competent Authority (DGAV) - ref. 2017_03.

The VCMSH2$^{LoxP/LoxP}$ strain used was kindly provided by the group of Prof Winfried Edelmann, under a MTA. This strain was introduced at the i3S facility in 2015 and rederived soon after arrival. This strain is characterized by tumor formation at the small intestine by the age of 9 months due to a conditional *Msh$_2$* mutation at the intestinal mucosa [275]. Animals were kept inside the barrier under SPF conditions and tested every 3 months for the following agents: MHV, EDIM, MPV, MVM, PVM, Sendai virus, TMEV, Ectromelia, LCMV, MAD-1 and 2, Mouse *Cytomegalovirus*, Reovirus, *Bordetella bronquiseptica*, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutsheri*, *Cryptosporidium* spp., *Mycoplasma pulmonis*, *Pasteurella* spp., *Pseudomonas aeruginosa*, *Salmonella* spp., *Helicobacter* spp., *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptobacillus moniliformis*, *Klebsiella pneumoniae*, ectoparasites (*Myobia, Radfordia* and *Myocoptes* spp.), *Eimeria* spp., *Entamoeba muris*, *Giardia* spp., *Spironucleus muris*, *Tritrichomonas muris*, *Aspiculuris tetraptera* and *Syphacea obvelata* and *muris*. Occasional positives for *Klebsiella* spp. and *Staphylococcus aureus* are found in the barrier zone and for these two agents plus *Helicobacter* spp., *Pasteurella* spp. and MNV at the experimental area. Water quality evaluation was performed every 3 months and environmental control was performed once a year. All animals were accommodated under the same conditions: animals were housed in groups of 6 to 10 animals in eurostandard type III cages with a filter top lid; distillated water was provided *ad libitum*, as well as autoclaved food (2014S diet from Envigo diets); corn cob was used as bedding material and all cages were provided with environmental enrichment consisting in two card tube roles and paper sheets as nesting material. Cages were changed once a week.

Husbandry practices at the barrier zone: all material was either autoclaved or decontaminated using hydrogen peroxide; a shower was required before access to the barrier rooms and personal protective equipment included a sterilized surgical suit, double pair of gloves, dedicated shoes, lab coat, hair cover, and surgical mask; all cages were manipulated inside a laminar flow; access was restricted to the facility caretakers, veterinarian, and technicians.

Husbandry practices at the experimental zone: experimental area followed the same rules concerning the material used inside the room (all the material used for animal husbandry was autoclaved); cages were open on a benchtop; access to the experimental area included researchers with access to the animal facility; personal protective equipment at the experimental area entry included shoe covers, lab coat, gloves and surgical mask, all sterilized.

The same procedures regarding cage changing frequency and other husbandry practices were applied in both areas.

A total of 31 VCMSH2$^{LoxP/LoxP}$ animals were used: 12 animals were kept at the barrier (7 males and 5 females) – MSH2 BAR group; 18 at the experimental area (9 males and 9 females) – MSH2 EXP group. 18 C57BL/6J animals were used as controls at the barrier zone (9 males and 9 females) (B6 BAR group) and another 18 animals inside the experimental area (9 males and 9 females) (B6 EXP group). Control groups were only used for inflammatory scores at the duodenum, lactoferrin, and $CD8^+$ and $CD4^+$ quantification. Microbiota analysis was only performed in the VCMSH2$^{LoxP/LoxP}$ group.

Animals were born either at the experimental area or at the barrier zone and were kept at these areas until 10 months of age. At this time point, all animals were euthanized using isoflurane overdose, followed by cervical dislocation. Duodenum, jejunum, and colon were collected and sent for routine histopathology evaluation. Feces from the colonic area were also collected at the final time point and frozen at -20ºC.

Feces collection for lactoferrin assays

Feces collection was performed at 10 months of age for lactoferrin measurement. Animals were individually housed for a few minutes in sterilized clean cages. Sterilized forceps were used to collect the samples individually and these were immediately used for ELISA assays in 1 ml Eppendorf® microcentrifuge tubes (Enzifarma, Portugal) according to the method described by Logsdon *et al* [297].

Microbiota analysis

Sample collection and DNA extraction

Mouse feces for microbiota analysis were collected during necropsy performed at 10 months of age, using 1 ml Eppendorf® microcentrifuge tubes (Enzifarma, Portugal) and frozen right after collection at -20ºC until further analysis. DNA extraction was performed using PureLink™ Microbiome DNA Purification Kit (Invitrogen) following the manufacturer instructions. Each sample consisted of 1-3 fecal pellets.

Real-Time quantitative polymerase chain reaction (qPCR)

The total amount of bacteria existing in each fecal sample was determined by qPCR using an universal qPCR assay that targets a conserved region of the 16S rRNA gene. qPCR reaction was prepared in a 10µL reactions containing 1× NZYSpeedy qPCR Probe ROX (NZYtech, Lisbon, Portugal), 1x Universal probe assay (Integrated DNA Technologies, Heverlee, Belgium) Microbial DNA-free water (Qiagen), and 1 µL of sample DNA. The qPCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following conditions: 3 min at 95ºC, followed by 35 cycles of denaturation at 95ºC for 10s and annealing/extension at 60ºC for 30 seconds. Two replicates were performed for each sample. PCR blanks were prepared by adding Microbial DNA-free water (Qiagen) instead of DNA. The relative standard curve method was used to quantify the total bacterial load present in the samples. The pGEM-T easy plasmid system (Promega, Madison, WI, USA) was cloned with a copy of the full 16S rRNA gene of *Escherichia coli* to create a standard sample of known concentration. Next, a series of standard samples were produced by diluting the plasmid of known copy number. A standard curve was created by plotting the log10 of copy number and the determined threshold cycle (Ct) value. Reaction parameters such as slope, y-intercept, correlation coefficient, and efficiency were extracted to control the calibration of the standard curve.

16S rRNA gene amplification and sequencing

16S rRNA gene was amplified using universal primers fused with Illumina adapters sequences U789F 5'-CGTCGGCAGCGTCAGATGTGTATAAGAGACAGTAGATACCCBDGTAGTCC-3' and U1053R 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTGACGACRRCCATGC-3' (Integrated DNA Technologies) that target the V5-V6 hypervariable regions. Amplification was performed in 35 µL reactions containing 1x AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA) and 0.4 µM of forward and reverse primers (Invitrogen, Foster City, CA, USA). PCR negative controls were prepared by adding Microbial DNA-free

water (Qiagen) instead of DNA. Amplicons underwent a purification step with magnetic beads using the Axy Prep PCR Clean-Up Kit (Axygen, Union City, CA, USA) to removed possible unspecific amplifications at lower molecular weights and visualized in 1.5% agarose gels. Purified PCR products were quantified using the Qubit dsDNA HS Assay Kit (Life Technologies, Foster City, CA, USA). Equal amounts of amplicons were used to prepare the sequencing library with the Illumina 16S Metagenomic Sequencing Library preparation protocol. The sequencing library was sequenced in an Illumina MiSeq platform using the MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA), with a read length of 300bp paired-end reads and an expected output of 100,000 reads per sample.

Sequencing data analysis

Raw paired-end reads were assembled and primers sequences were removed at both ends. Then, reads were quality filtered by imposing a maximum number of expected errors of 1.0 and were globally trimmed to a fixed length. Filtered reads were dereplicated and OTUs were clustered using the UNOISE algorithm [298, 299], which identify accurate biological sequences and filters chimeric sequences. Operational taxonomic units were aligned against the 18S rRNA (SILVA 18S rRNA v123) and the internal transcribed spacer (RDP ITS v2) sequences to identify putative eukaryotic contaminations. An OTU table was constructed by mapping reads back to OTUs to get counts per sample. The OTU table was rarefied to a fixed number of reads (37,000 reads) to control differences between samples. Next, each biological sequence was taxonomically assigned with the SINTAX algorithm [300] using the 16S RDP Classifier training set v16 as the reference database. Alpha-diversity was determined using the Shannon index, Chao1 estimator. Beta-diversity was assessed by the weighted and unweighted UniFrac distance [301]. Differences in beta-diversity were calculated in distance matrices and visualized in boxplots. Sequencing data analysis was performed using usearch_v8.1.11861_i86linux64, usearch11.0.667_i86linux32 and QIIME v1.9 [302].

Histopathological analysis and Immunohistochemistry

Tissue samples were cut to 4um thick and then stained using hematoxylin-eosin before histopathology analysis. Inflammatory levels at the small intestine and colon were characterized using a score for inflammatory bowel disease described by Erben *et al* [303]. For immunohistochemistry, duodenum tissue sections were first deparaffinized, hydrated, and then treated with 1× citrate buffer (pH 6.0) or 1x EDTA buffer (pH 9.0) (Dako) for 40 min at 100 °C. All of the following steps were performed at room temperature (RT). Unspecific endogenous peroxidase activity was eliminated with 3 % hydrogen peroxidase in methanol for 20 min. To reduce nonspecific background staining, slides were blocked with Ultra V

Block (Thermo Scientific) for 10 min. Slides were rinsed in PBS–0.1 % Tween20 and incubated overnight (ON) with the antibody anti-CD4 [EPR19514] (Abcam) diluted 1:500 or incubated ON with anti-CD8 [EPR20305] (Abcam) diluted 1:2000 in UltraAB Diluent (Thermo Scientific). Slides were then incubated with Dako Real EnVision HRP Rabbit/Mouse solution (Dako) for 30 min. Slides were washed, developed for 1–3 min with 2 % Dako REAL™ DAB+ Chromogen solution (Dako), counterstained with haematoxylin, dehydrated, and mounted with mounting medium (Thermo Scientific). All washing steps were performed in PBS–0.1 % Tween20 buffer. Negative controls were obtained by substituting the primary antibody with immunoglobulins of the same class and concentration and positive controls were performed in lymphoid zones. Slides were blind-reviewed by two independent researchers, and the percentage of positive cells was quantified using ImageJ software (NIH).

Statistical analysis

GraphPad Prism version 8.02, (GraphPad Software, La Jolla, CA, USA), was used for statistical analysis. The normality of the data was evaluated with D'Agostino Pearson omnibus and Shapiro-Wilk. Differences between two groups of samples were evaluated by Student's t-test if data follows Gaussian distribution or by Mann-Whitney if data follows a non-normal distribution. $CD8^+$ T cells levels were analysed using a two-way ANOVA test. $CD4^+$ T cells were compared using a Kruskal-Wallis test. Lactoferrin values where compared using a Mann-Whitney test. Differential taxonomic analysis was performed using linear discriminant analysis (LDA) effect size (LEfSe) [304]. Only significant taxa with a LDA greater than 2 were considered. Statistical differences in UniFrac distance matrices between groups of samples were evaluated with analysis of similarities (ANOSIM). All statistical tests performed were two-sided, and differences were considered significant at *P*-values lower than 0.05.

Results

Phenotypic characterization of VCMSH2$^{LoxP/LoxP}$ animals in the barrier and in the experimental areas

Microscopic evaluation was used to evaluate the levels of inflammation in the small intestine (duodenum and jejunum) and in the large intestine (distal colon), and to identify tumors at the small intestine at 10 months of age. In the barrier area, 7 of the 12 MSH2 BAR animals developed tumors (*in situ* carcinoma or well-differentiated carcinoma), an incidence of 58%. Of those, 3 animals had tumors in the duodenum, 2 had tumors in the jejunum, and the remaining 2 had tumors in both locations (table 1). In the experimental area, 7 out of the 18 MSH2 EXP animals presented tumors (38%); one animal developed a tumor in the

duodenum, 5 in the jejunum, and one animal had tumors in both locations. No statistically significant differences were found between the two areas of the animal facility regarding the number of animals with tumors or between the number of tumors found in the duodenum and jejunum (P = 0.242 and P = 0.201, respectively; Table 1).

Considering the inflammatory scores of mice intestinal mucosa according to the housing area, in the jejunum, a significantly lower score in MSH2 BAR mice was detected in comparison with MSH2 EXP (P = 0.0145). A significant difference was also found between the inflammatory scores of the colon between MSH2 BAR and MSH2 EXP (P = 0.0061).

Table 1. Tumor incidence and inflammatory scores for the different groups of animals according to the housing area

Strain	Anatomical area	Tumor incidence	Inflammation score (Mean±SD)
MSH2 BAR (N=12)	Global	7 (58%)	
	Duodenum	5 (42%)	7±2.4
	Jejunum	4 (33%)	3.6±1.9
	Colon	0	2.3±1.2
MSH2 EXP (N=18)	Global	7 (38%)	
	Duodenum	2 (11%)	7.5±3.9
	Jejunum	6 (33%)	5.6±2.5
	Colon	0	3.9±2.9
B6 BAR (n=18)	Duodenum	0	7.6±3.4
B6 EXP (n=18)	Duodenum	0	8.6±3.9

Table 2. Inflammation scores, lactoferrin, $CD8^+$ and $CD4^+$ T cells in MSH2 groups according to the presence of tumors

		MSH2 BAR (n = 12)		MSH2 EXP (n = 18)		B6 BAR	B6 EXP
		With tumors	Without tumors	With tumors	Without tumors		
N total (%)		7 (58%)	5 (42%)	7 (38%)	11 (62%)	18	18
Inflammation score (mean ± SD)	**Duodenum**	7 ± 2.4 (n=5)	7 ± 2.7 (n=7)	5.9 ± 3.1 (n=2)	8.5 ± 4.2 (n=16)	7.6 ± 3.4	8.6 ± 3.9
	Jejunum	4.6 ± 1.6 (n=4)	2.2 ± 1.5 (n=8)	4.9 ± 1.3 (n=6)	6.3 ± 3 (n=12)	-	-
Lactoferrin (ng/g feces)		1.5 ± 1.8	1.3 ± 1.3	3.2 ± 3.2	3.6 ± 3.6	1.5 ± 1.1	1.1 ± 0.4
CD4+ T cells (duodenum)		9.5 ± 3.2	6.3 ± 3.6	6.8 ± 2	10.1 ± 9.1	4.8 ± 4.7	6.4 ± 5.4
CD8+ T cells (duodenum)		17.2 ± 7.4	17.0 ± 7.1	11.3 ± 3.2	12.9 ± 2.8	15.8 ± 5.5	13.7 ± 4.2

When comparing the inflammatory score of MSH2 mice with and without tumors in the different areas of the small intestine, a significant decrease difference was identified in the jejunum score between animals with and without tumor accommodate in the barrier area (*P* = 0.0391). Data are summarized in tables 1 and 2.

Immunohistochemistry analysis for $CD4^+$ T cells and $CD8^+$ T cells revealed significant differences in the number of $CD8^+$ T cells between MSH2 BAR and MSH2 EXP *(P* = 0.0064). No differences between the two B6 control groups at the barrier and at the experimental area, or between animals with or without tumors, were found regarding $CD4^+$ and $CD8^+$ T cells (Figure 1).

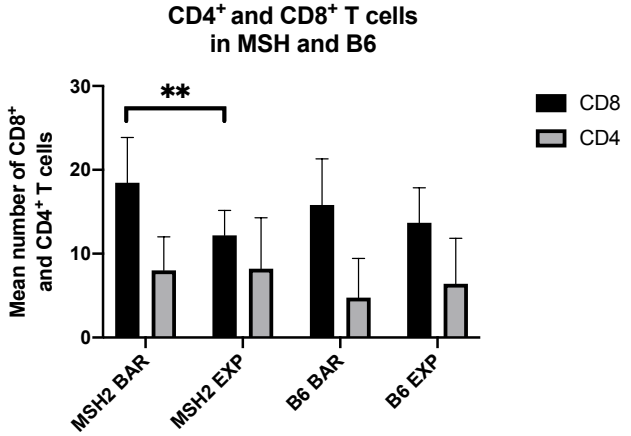

Figure 1. $CD8^+$ and $CD4^+$ T cells infiltrate in MSH2 and B6 mice at the barrier and experimental area.

Lactoferrin has emerged as a potential marker for monitorization of intestinal inflammation and can be assessed in a non-invasive way [305, 306]. At 10 months of age, lactoferrin levels were significantly higher in the MSH2 EXP mice (3.4 ± 3.4) than in the MSH2 BAR animals (1.4 ± 1.5; $P = 0.0214$). A significant difference in lactoferrin levels was also found between MSH2 EXP and the control group (B6 EXP; $P = 0.0079$). No significant differences between B6 BAR and B6 EXP were found ($P = 0.5799$; Figure 2 A).

Considering lactoferrin values in animals with tumors, no significant values were achieved in animals with or without tumor, neither in the barrier nor in the experimental area ($P = 0.9192$ and $P > 0.9999$ for barrier and experimental area groups, respectively). The comparison of lactoferrin levels between MSH2 BAR animals with tumor and MSH2 EXP with tumor was also not significant ($P = 0.1649$; Figure 2 B).

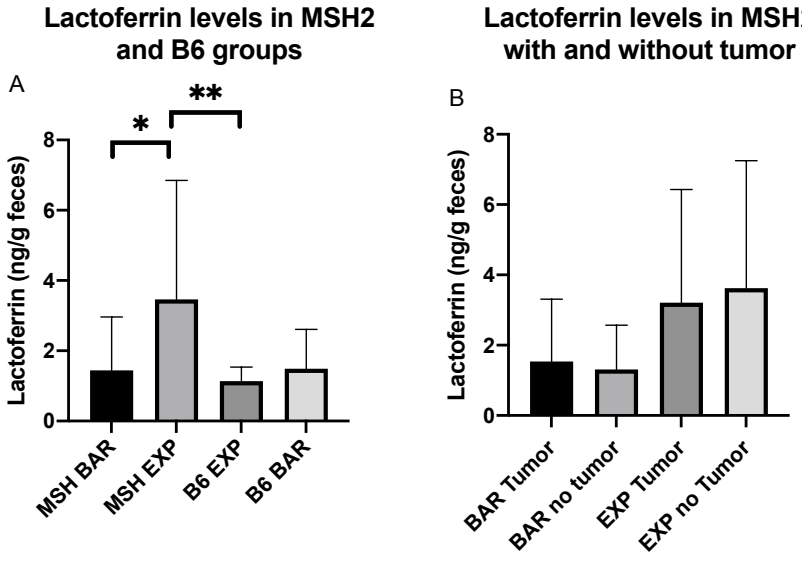

Figure 2. Lactoferrin levels between the 4 groups (A) and between MSH2 animals with and without tumor (B).

Gut microbiota profiling of VCMSH2$^{LoxP/LoxP}$ mice

The gut microbiota was compared between VCMSH2$^{LoxP/LoxP}$ mice housed at the experimental (MSH2 EXP) and barrier (MSH2 BAR) zones. The mean number of raw reads was similar between the two animal groups, with MSH2 BAR having 70,340 reads and MSH2 EXP having 63,597 reads ($P = 0.2521$). After fixing the number of reads to 37,000 per sample, no significant differences in the number of OTUs was found between the two groups ($P = 0.3347$).

To measure differences in the structure of the microbiota between animals in the two zones, we measured alpha diversity (diversity within samples) and beta-diversity (diversity between samples). Alpha-diversity measured using the Shannon index revealed a

significant decrease in gut bacterial diversity in MSH2 EXP animals in comparison with MSH2 BAR animals (P = 0.0036). These differences were associated with a significantly lower evenness in MSH2 EXP (P = 0.0008), and not by differences in species richness (P = 0.5530; Figure 3 A-C).

Beta diversity was measured using unweighted (quantitative) and weighted (qualitative) UniFrac phylogenetic distance matrices and visualized in PCoA plots. The gut microbiota structure was significantly different between the two groups of animals (ANOSIM R = 0.5078, P < 0.0001 and ANOSIM R = 0.384, P < 0.0001, for unweighted and weighted Unifrac, respectively; Figure 3D-E). However, no significant differences were found in the inter-individual distance between animals of each zone (P = 0.9768 and 0.0635, respectively for unweighted and weighted values; Figure 3 F-G).

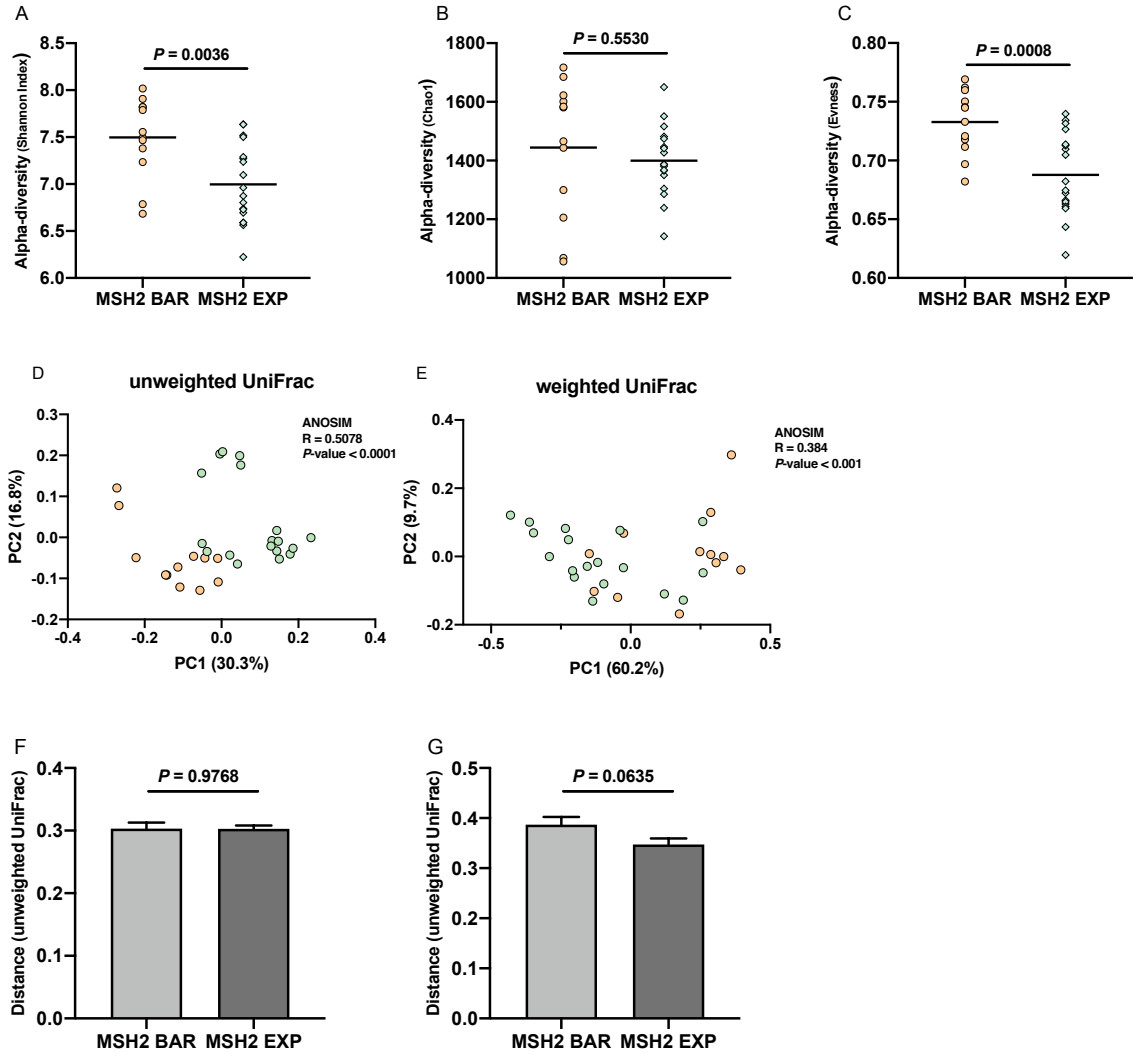

Figure 3. Microbiota characterization of MSH2 mice feces. (A) Shannon index for MSH2 BAR and EXP animals. (B) Estimated number of species (richness) in the two groups. (C) Abundance of species in the two groups (evenness). (D) unweighted and (E) weighted UniFrac analysis for beta diversity. Inter-individual distance within animals of each group. (F) unweighted and (G) weighted.

The two main phyla recognized in the gut microbiota of VCMSH2$^{LoxP/LoxP}$ mice were Firmicutes and Bacteroidetes. Bacteroidetes were the most abundant phylum in MSH2 BAR (50.1%) being significantly overrepresented in these animals in comparison with MSH2 EXP (32.6%; *P* = 0.005; Figure 4 A and B). Unclassified Bacteroidales were the most representative taxa within the Bacteroidetes phylum. In contrast, Firmicutes were the most prevalent phylum in MSH2 EXP (61.7%) and were also significantly overrepresented in comparison with MSH2 BAR (42.2%; P=0.0116). *Lachnospiraceae* and *Lactobacillaceae*, including the *Lactobacillus* genus, were the most representative taxa within Firmicutes (Figure 4 C and D).

Additionally, significant differences between *Verrucomicrobia* (P = 0.0056) and other unclassified bacteria (P = 0.0441) were also found.

To identify the taxa that might explain the differences between animals in the barrier and experimental areas, a taxonomic differential analysis (using LEfSe [304]) was performed. This analysis identified significant differences in 30 taxa, including 21 taxa enriched in MSH2 BAR and 9 taxa enriched in MSH2 EXP mice (Figure 5). The gut microbiota of MSH2 BAR was enriched in eight bacteria genera namely, *Akkermansia* sp., *Enterorhabdus* sp., *Staphylococcus* sp., *Ruminococcus* sp., *Prevotella* sp., *Butyrivibrio* sp., *Parabacteroides* sp. and *Acetatifactor* sp., while the gut microbial community of MSH2 EXP mice was enriched in six genera including, *Fusicatenibacter* sp., *Gordonibacter* sp., *Eubacterium* sp., *Parvibacter* sp., *Clostridium* sp., and *Desulfovibrio* sp. (Figure 5).

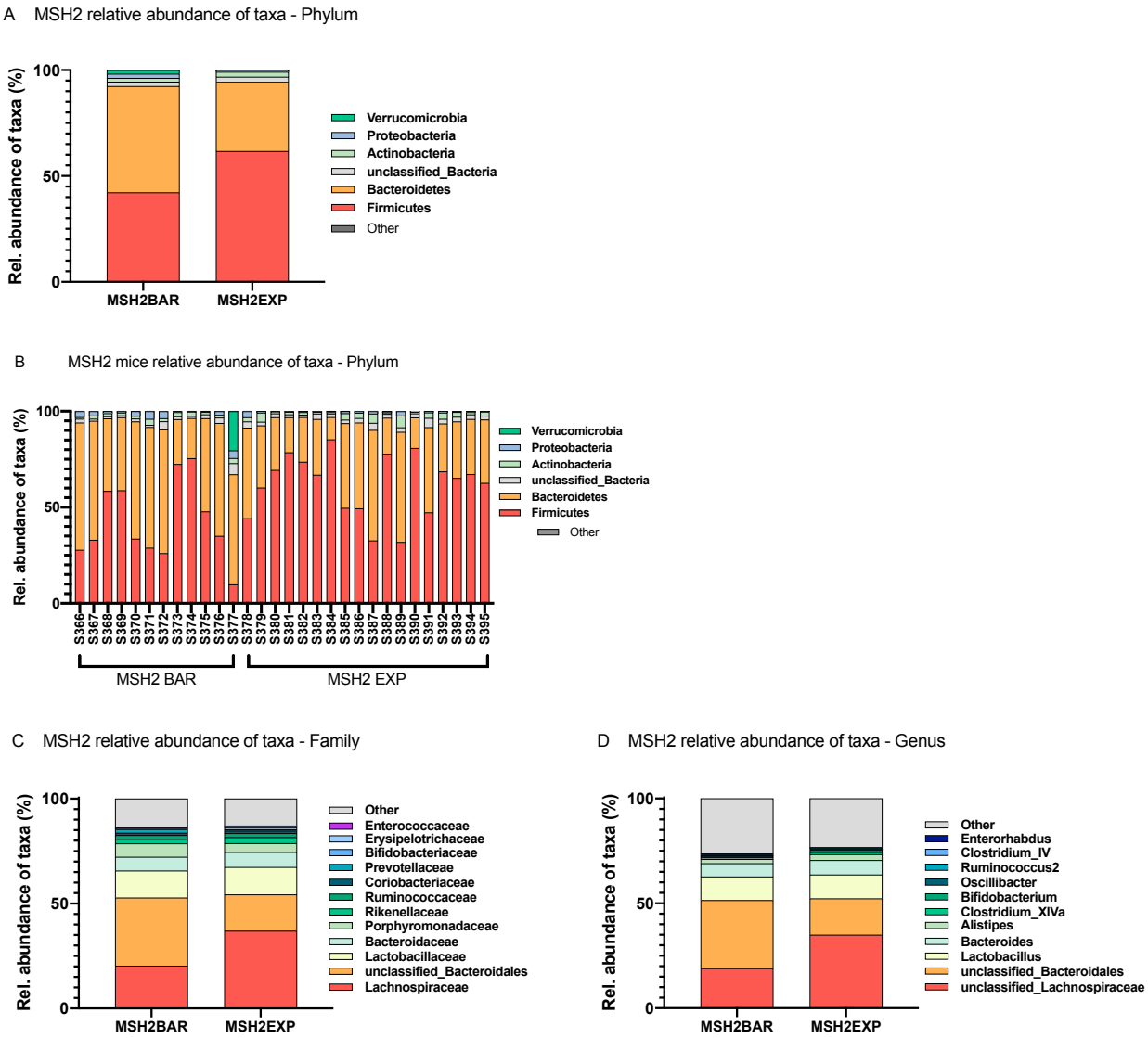

Figure 4. Relative abundance of phyla in MSH2 BAR and MSH2 EXP mice (A) and in individual mice (B). Relative abundance of families (C) and genera (D) in MSH2 BAR and MSH2 EXP mice.

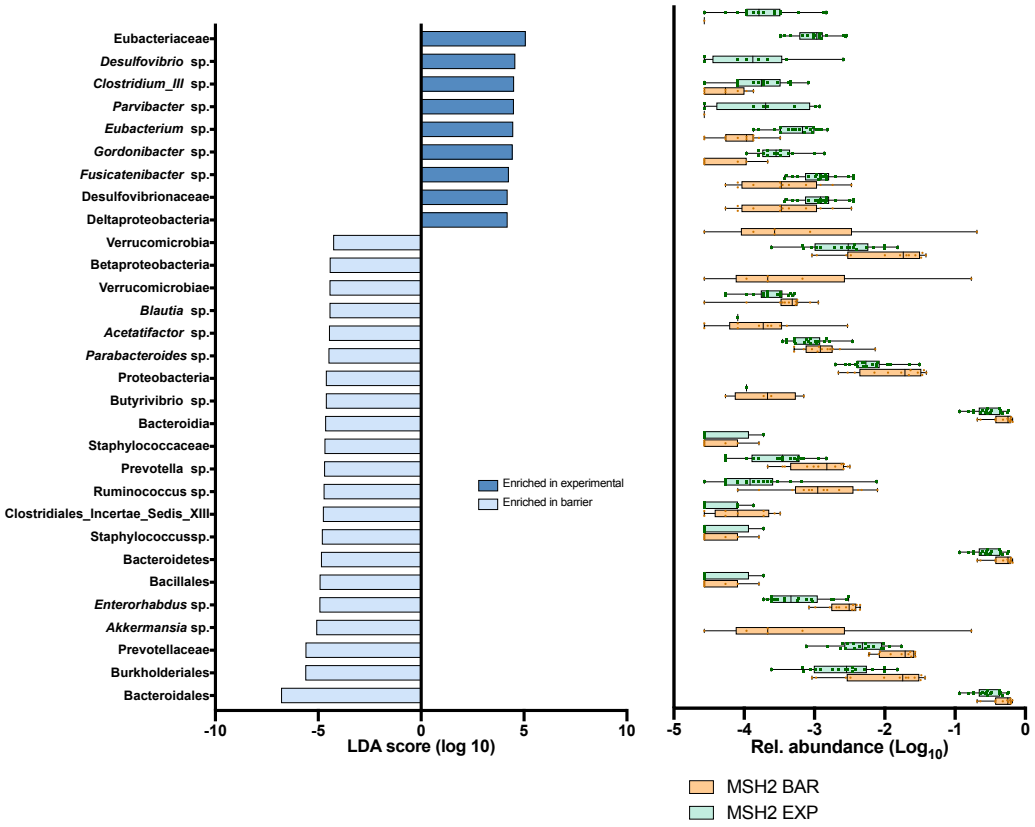

Figure 5. Differential taxonomic analysis showing the LDA score of the LEfSe analysis and the differences in the relative abundance of enriched taxa between MSH2 BAR and MSH2 EXP mice.

Relationships between the gut microbiota and the phenotypes of VCMSH2$^{LoxP/LoxP}$ mice

Correlation analysis was performed to evaluate possible relationships between the gut microbiota and mice phenotypes in both facility areas.

When considering tumor development in MSH2 BAR and in MSH2 EXP mice, no significant correlations were observed with the gut microbial alpha diversity (P = 0.6494 and P = 0.2678, respectively). Likewise, the beta-diversity was not correlated with tumor development in the MSH2 BAR nor in the MSH2 EXP mice (P = 0.5075 and 0.1748, respectively).

Regarding inflammatory phenotypes, the duodenal inflammatory score was positively correlated with the alpha diversity (Shannon index) in MSH2 BAR animals. In this group of animals, there was a negative correlation between the jejunum inflammatory score and the abundance of *Prevotellaceae* and a positive correlation with the abundance of *Clostridiales_Incertae_Sedis_XIII*. Additionally, in MSH2 BAR mice, the colon inflammatory score was positively correlated with the abundance of *Bacteroidales*, *Bacteroidetes*, *Ruminococcus* and *Bacteroidia*. The levels of lactoferrin in MSH2 BAR mice were negatively correlated with the abundance of *Enterorhabdus* sp. and *Fusicatenibacter* sp. (both P =

0.03). There were no significant correlations between inflammatory phenotypes and the gut microbiota in MSH2 EXP animals.

Discussion

The stability of gut microbiota is an important factor when planning animal experiments. Ignoring this variable has the potential to change experimental results, reduce reproducibility, and increase the number of animals needed for a given experiment. Several strategies and variable factors can be adopted to reduce variability, such as the diet, husbandry practices, or rederivation techniques [4]. Knowledge about the possible impact that microbiota may have in a specific model of disease can help to decide how to manage the microbiota variable [296]. It is known that the microbiota can vary between different mice strains, animals with different origins, and even between animals housed in distinct rooms of the same facility. Thus, in the current investigation, we aimed to further characterize a mouse model of cancer ($VCMSH2^{loxp/loxp}$) housed in two different areas of the facility. These areas are characterized by two distinct microbiological units: a barrier area with restricted movements and an experimental area where a higher number of users can have access to animal rooms. In order to characterize these models, animals were submitted to fecal collection at 10 months of age for lactoferrin assays and microbiota profiling (distal colon) and tissue collection for quantification of $CD8^+$ and $CD4^+$ T cells, to the determination of tumor incidence and inflammatory levels.

Distinct phenotypes were observed in the barrier and in the experimental areas of the animal facility. At 10 months of age, $VCMSH2^{LoxP/LoxP}$ mice presented different tumor incidence between the barrier (58%) and the experimental area (38%), although this difference was not statistically significant. The same is applied to the tumors location: in the experimental area, animals revealed a higher percentage of tumors in the jejunum, thus these differences were also not statistically relevant. Previous reports only characterized the presence of tumors at the small intestine, without considering the duodenum or jejunum topographies [275]. Nevertheless, only representative portions of duodenum and jejunum were analyzed in this study, which can represent a limitation. A more thorough screening using other sample preparation techniques (such as the swiss role) could add more precise information.

When comparing the inflammatory scores in MSH2 animals, similar values were obtained in the duodenum, nonetheless the animals in the experimental area revealed higher scores in jejunum and colon. This could be related to the presence of pathogenic agents, as occasional positives for *Helicobacter* sp. or *Pasteurella* sp. are found in the experimental area. The presence of tumors, however, was not related to the levels of inflammation, except for the barrier group in which inflammatory scores at the jejunum were higher in

those animals that developed tumors. These results are contradictory with the higher levels of $CD8^+$ T cells observed in the duodenum of the animals raised in the barrier area, despite the lack of difference in inflammatory scores in this region. Further characterization of $CD8^+$ and $CD4^+$ T cells is required to allow comparison between these values and the scores obtained in the jejunum and colon. We could not establish any relationship between the levels of $CD8^+$ and $CD4^+$ T cells and the gut microbiota profile. One possible explanation for these observations is that T cell immunohistochemistry was performed in the duodenum, whereas the gut microbiota was characterized in feces, which may reflect the distal colon area. $CD8^+$ T cells have an important role in the response against cancer cells and its role in immunotherapy is known [274]. Tumors from MSH2 mice present little or no inflammatory infiltrates and thus, the variation in the $CD8^+$ T cells may be related to other variables independent from the tumor formation.

Lactoferrin is a glycoprotein found in the secondary granules of neutrophils and has an important anti-inflammatory role in infectious diseases [307] and potential to reduce inflammation in dextran sulfate sodium treated animals [308]. Lactoferrin was also described as a possible biomarker for colorectal diseases [309] and in animal models, it can be used as a non-invasive tool to monitor intestinal inflammation in an infectious disease context [297]. In this study, lactoferrin levels at 10 months of age were significantly higher at the MSH2 EXP animals, but no relationship could be established between lactoferrin levels and tumor development. Although lactoferrin was described as a biomarker for colorectal disorders [309], in this particular mouse model where the tumors occur at the small intestine, we could not find any evidence of increased lactoferrin levels in animals with tumors.

The differences found in $CD8^+$ T cells, lactoferrin levels and the inflammatory scores from the jejunum and colon areas can, in part, be related to different microbiota as the two areas of the animal facility are considered two distinct microbiological units. The duodenum and the jejunum have a characteristic microbiota [244] and since only colon feces were evaluated, no further inferences will be reasonable to make. Despite that, alpha-diversity was significantly different between MSH2 mice at the barrier and at the experimental area, mainly due to differences in the abundance (evenness) of certain microorganisms. This was reinforced by the beta-diversity results that showed a distinction of the gut microbial communities of mice in the two distinct areas. The dominant phyla were similar to the already described for mice, Bacteroidetes and Firmicutes [310], but housing these mice in two distinct areas has inverted the abundance of these microorganisms: Firmicutes were increased in the experimental area and Bacteroidetes were dominant in the barrier. A decrease in Firmicutes has been previously associated with a reduction in the number of polyps in $APC^{Min/+}MSH2^{-/-}$, due to a reduction of butyrate forming bacteria [276], but this

finding was not replicated in this VCMSH2$^{LoxP/LoxP}$ model. However, these are two different mouse models and different methods for gut microbiota manipulation were used, which refrains a thorough comparison. It was expected that animals in the experimental area, which detains a higher flux of users, reagents, and equipment would have a higher richness in microorganisms, which was not evident in this case.

The correlations found between inflammatory scores observed in the duodenum and jejunum in mice raised in the barrier area and the microbiota may not reflect a causal effect, as the inflammatory score corresponds to a distinct anatomical area from where the feces were collected. The same correlations were not significant in the MSH2 EXP animals. Levels of $CD8^+$ and $CD4^+$ T cells were also not correlated with any specific alteration in the relative abundance of different taxa but, again, microbiota was not representative of these specific areas. When analyzing the relationship between the gut microbiota and the lactoferrin levels in the barrier area (MSH2 BAR group) at 10 months, a negative correlation was found between this variable and the presence of *Enterorhabdus* and *Fusicatenibacter*. This last microorganism was already described as being decreased in patients with ulcerative colitis [311], which can be a reflex of higher inflammatory levels in animals with higher levels of lactoferrin. We could not find any valid correlation between the lactoferrin levels and the inflammatory scores at 10 months of age.

This study was focused on a single timepoint (10 months), lacking a longitudinal characterization of the different parameters. The microbiota is a dynamic variable throughout the animal life [312]. Early life exposition to different microorganisms will shape the immune system but a small period of time is critical to determine the way the immune system will behave in the future [211, 313]. Tumor growth is equally determined by the gut microbiota as highlighted by antibiotic treated animals [314]. Treatment of mice with antibiotics early in life can delay the onset of tumor growth in genetically modified animals with tumor predisposition and differences in the abundance of specific microorganisms are correlated with tumor growth, despite the similar microbiota at early life between predisposed and wild type animals [315].

Regardless the limitations of this study, we can conclude that implementing similar rules for personal protective equipment and sterilization of consumables in contact with animals can minimize the differences in the gut microbiota of mice but still, differences will occur. The VCMSH2$^{LoxP/LoxP}$ model resulted in different levels of lactoferrin, $CD8^+$ T cells, microbiota and inflammatory scores. These differences should be carefully considered when distinct microbiological units are used for mice maintenance. The particularities found in this work do have impact in the experimental results and may produce undesired variability and difficulties in data interpretation and results reproducibility.

General discussion

Assisted reproductive techniques are commonly used in laboratory rodent facilities as a tool to improve reproductive performance, reduce animal use and control the microbiological content of animals. Assisted reproductive techniques are also a powerful tool to facilitate the exchange of animals between facilities.

The techniques considered in this investigation, mainly mouse superovulation, incubation and embryo transfer, presented a significant and positive impact on the application of the 3Rs, especially on the reduction of animal numbers. These techniques are the basis for the application of other important protocols, such as the production of genetically modified mice, rederivation techniques and cryopreservation.

Optimal conditions were defined by the different steps explored in this investigation, especially for C57BL/6J mice. Considering the protocol for superovulation and embryo transfer in B6, superovulation had the better outcome in the number of oocytes produced when 3 weeks females were used for superovulation at 7 pm (at the same time the room lights were off). After superovulation, B6 embryos tend to have a higher survival rate if collection of embryos is performed at 0.5 dpc and incubation in an optimal medium is used to allow the passage to a 2-cells stage. In this particular strain, the final step required for a litter production, embryo transfer, was related to higher success rate when only 6 embryos were used in unilateral transfers in CD-1 recipient females. Although the first step of superovulation was not explored for other strains, GMM strains at a B6129 background also had better outcomes when embryos were collected at 0.5 dpc after superovulation and incubated up to a 2-cells stage. Embryo transfer in B6129F1 produced optimal results when 12 embryos are transferred unilaterally or when 6 embryos were used for bilateral transfer.

Superovulation protocols were, in the past, optimized regarding age, estrous cycle phase, weight and the timing of hormone administration. Our work analyzed the results of females superovulated at 3, 4 and 5 weeks of age, using the same hormone dose but at 3 different schedules (3, 5 and 7 pm with a light cycle between 7 am and 7 pm). The total number of oocytes per female was in this way analyzed and allowed us to conclude that age and schedule are significant factors on what is concerned to superovulation, as well as the interaction between these two factors. Surprisingly, weight and the estrous cycle did not result as significant factors. C57BL/6J females produced significantly more oocytes when 3 weeks females were used at 3 and 7 pm, this last schedule with better results. When considering only the female's age, 3 weeks females produced better results and in fact, the number of oocytes produced by 3 weeks females at 3 pm and 7 pm were very similar. However, when females at 4 and 5 weeks of age were used, better results were obtained in the 7 pm group and, even so, lower number of oocytes were produced by these two ages

at 3 and 7 pm when compared to females at 3 weeks of age. The 3 ages used for this experiment were already described as optimal for superovulation in B6 mice by other authors [60, 63] but herein it was emphasized by the fact that older females can have optimal results if superovulation is done at 7 pm. These results lack, however, the evaluation of fertilization levels and embryo quality.

Superovulation has been described to have optimal results when hCG is given a few hours before the LH surge [71], which is consistent with the results obtained for 3 weeks females at the 3 pm schedule. But females at 4 and 5 weeks, produced higher number of oocytes when the hormone administration was closer to the internal LH surge. The timing of ovulation seems to be dependent on the midpoint of the dark cycle and the LH surge is described to occur in the afternoon/evening, or 15 to 20 hours after the midpoint of the dark cycle [51, 71]. No information is available regarding LH secretion in mice at different ages. One possible explanation to this result is that LH and estrogen production before the LH surge may vary according to the female's age and females at 4 and 5 weeks of age may have different sensibility to PMSG and hCG administration and thus better results in only one of the schedules. The onset of puberty may also explain this result as females at 4 and 5 weeks of age are closer to this event. This can possibly mean that in females were the puberty has already been settled, synchronizing the hormone administration with the internal LH surge seems to be more efficient. These results also allow for a reduction on the number of animals used, especially if females at the onset of puberty are used.

The second experiment, exploring superovulation effects, analyzed the impact of this protocol on the number of embryos obtained per female and aimed to optimize the ideal timing for embryo collection. The negative effects of superovulation are characterized and its effect on the quality of embryos and on the final number of embryos or pups obtained is known [316, 317]. We analyzed the impact of maintaining the embryos in the superovulated female or collecting the embryos sooner and allowing embryo development in optimal incubation conditions. Historical data from 3 GMM strains, grouped according to the genetic background (C57BL/6J, B6*129 and FVB/NJ) was analyzed to compare the number of two-cells embryos obtained when collection is performed at 1.5 dpc after female superovulation and mating, and the number of two-cells obtained after collection of 0.5 dpc embryos submitted to KSOM incubation. The same experiment was repeated in wild type C57BL/6J, aiming for a less variable set of results. This experiment allowed us to conclude that collecting the embryos at 0.5 dpc and favoring their passage to a two-cells stage inside the incubator significantly increased the number of two-cells embryos obtained in C57BL/6J and B6*129. GMM strains at an FVB/NJ background, produce equally more two-cells embryos when incubation is used, although we could not find a statistically relevant difference. The experiment using C57BL/6J, wild type, resulted in a significant difference

between the two collection conditions and a higher number of two-cells embryos when incubation was used. As already discussed in the introduction, superovulation is known for having impact on embryo quality. This is reflected in several epigenetic and DNA alterations [76] but also on the subsequent pregnancy and embryo development, impairing placental [15] and embryo development [85, 86], and increasing the frequency of premature births and low weight at birth [83, 89]. The better results obtained after incubation may be related to the fact that, when embryos are collected at 0.5 dpc, the time of exposure to detrimental doses of PMSG and hCG is reduced. The half-life of PMSG and hCG is longer than FSH and LH half-lives [90], meaning that when superovulation is used, the embryos are not only exposed to a higher dose of PMSG and hCG but also for a longer period of time. It is expectable that this exposition may be detrimental for the embryo development and, for that reason, collecting the embryos sooner may reduce the exposition to PMSG and hCG. This outcome, also explored by other authors, but at a latter phase of embryo development [86], shows that superovulation negatively impacts embryo development and that early collection of embryos seems to refrain the negative impact of superovulation. Despite the fact that the quality of embryo's was not evaluated, the results of this research promote a reduction in the number of females used for embryo collection. Significantly lower number of females can be used if collection is performed at 0.5 dpc followed by incubation or transfer when compared to later times of collection. Many protocols require the collection of 2-cells embryos but, methods described for embryo collection vary. Thus, this information may allow for a higher efficiency of embryo collection as embryo loss seems to occur, at least for the strains analyzed, at a very early phase of development.

The last step in many protocols requiring embryo manipulation, embryo transfer, was equally investigated using different surgical conditions, number of embryos and reciprocity between receptive female and embryos as many variations are described for this technique. This experiment included unilateral and bilateral transfers of B6 and B6*129 embryos, using 6, 8, 12, 15, 20 and 25 unilateral and 6, 12 and 20 embryos bilateral for B6 and 6,12 and 20 unilateral and bilateral for B6*129. Reciprocity was tested using B6 embryos and B6 receptive females with 6 embryos.

These experiments allowed for the first time to compare different number of embryos using wild type mice. Moreover, information regarding bilateral transfers was only available for large number of embryos. As already stated, 6 embryos unilaterally produced the best outcome in B6. This first outcome was in accordance with previous published data, stating that lower number of embryos are related to higher success rates [96] and it can significantly impact the number of donor females needed for rederivation purposes as the effort measured as the number of embryos needed to produce one live pup, is lower when 6 embryos are used per female (1.3 embryos to produce one live pup). The limitation when

larger number of embryos are used unilaterally may be related to the uterine capacity, already described by Johnson *et al* [151], corresponding to about 11-12 embryos, but also to anatomical features such as the double cervix that avoids embryo migration between uterine horns [318]. Similar results were found for a hybrid strain (B6129F1) when using unilateral transfers but some strain related differences were detected when bilateral transfers were used. Unilateral transfers using only 6 embryos in B6129F1 resulted in a slightly better success rate when compared to the same condition using B6 embryos. For B6, bilateral transfers were related to higher mean number of pups and higher success rates when larger number of embryos were used but, this result was not reproduced in the B6129F1 experiment where low number of embryos produced higher success rates. For bilateral transfers it seems that a minimum number of pups is required per uterine horn to establish a successful pregnancy. Bilateral transfers using 6 embryos in B6 was the condition that produced the lowest mean number of pups, which can be explained by strain variability. B6*129F1 bilateral transfers using 20 embryos had lower success rates when compared to the same condition in B6 and the larger size of B6*129F1 may explain the inability of this last strain to develop a successful pregnancy with large number of embryos in the same uterine horn [319]. Together, these results can have a very positive impact on the optimal use of embryos and ultimately in reducing the number of donor females.

Due to the important impact that embryo transfer has in the GM definition, and the GM potential to influence experiments, we investigate the phenotype of a mouse model of small intestine cancer (the VCMSH2$^{LoxP/LoxP}$ mice). Characterized by tumor formation at the small intestine, the phenotype of these mice and control animals (C57BL/6J) was evaluated in two distinct microbiological units of the facility, the experimental and the barrier zone. In both facility compartments, this model of genetic predisposition for tumor development in the small intestine presented similar number of animals developing tumor at 10 months of age (58% at the barrier versus 38% at the experimental area, not statistically different). Additionally, differences in CD8$^+$ T cells infiltrates, levels of lactoferrin in feces, inflammatory scores in the small and large intestine and also in the alpha diversity of the GM were detected when both groups were evaluated. VCMSH2$^{LoxP/LoxP}$ maintained inside the barrier zone showed tumor formation in equal proportions in the duodenum and in the jejunum area but, the same model maintained in the experimental area revealed very little number of animals with tumors in the duodenum, and a higher number of animals with tumor in the jejunum (although not statistically different). Lactoferrin values were significantly higher at the VCMSH2$^{LoxP/LoxP}$ mice maintained at the experimental area compared to the barrier group. The inflammatory score of VCMSH2$^{LoxP/LoxP}$ mice was higher in the experimental area, but only for the duodenum and colon regions and the CD8$^+$ T cells at the duodenum were increased in VCMSH2$^{LoxP/LoxP}$ mice at the barrier zone. Despite that increase, no

differences in $CD4^+$ T cells were found. The GM of these mice revealed differences in alpha diversity (Shannon index) mainly related to differences in the abundance of certain microorganisms (evenness). Beta-diversity results also pointed a differentiation of mice by the two distinct areas. Mice maintained inside the barrier had an increased prevalence of Bacteroidetes and, at the experimental area, Firmicutes were the dominant phylum. Lactoferrin levels in the barrier were negatively correlated with *Enterorhabdus sp.* and *Fusicatenibacter sp.* This last microorganism, described as being decreased in patients with ulcerative colitis [311] may reflect the higher levels of inflammation measured through lactoferrin. Despite the characterization performed, we were unable to establish any valid correlation between the GM and presence or absence of tumors and the same is applicable to the possible interactions between the presence or absence of tumors and the other variables studied. Further important work regarding $CD4^+$ and $CD8^+$ T cells quantification in the other intestinal portions (duodenum and colon), as well as a more refined tumor characterization (number and size of the neoplastic lesions developed, their histopathological classification and molecular properties) will be conducted in order to accurately complete this study.

Finding differences in this model of genetic predisposition emphasizes the importance of microbiological control when experimental mice are used and the variability that may be introduced through GM. The GM can be changed even when slight changes in husbandry practices are introduced, promoting the alteration of different parameters with impaired future reproducibility of experiments and increasing variability.

Overall, the results herein achieved may contribute for substantial animal reduction, further optimization of different ART techniques and raise scientific awareness regarding the impact that the GM displays in disease models.

Final remarks

The current investigation consisted in different experiments based on mouse superovulation, timing of embryo collection and embryo transfer procedures and resulted in the following main outcomes:

- C57BL/6J mice superovulation should be performed closer to the beginning of the light cycle and in females at 3 weeks of age. Females at 4 and 5 weeks of age, also described as optimal for superovulation, had better outcomes in the same schedule, but the number of oocytes was lower when compared to 3 weeks females;
- An interaction between age and schedule was identified as being determinant for oocytes yields in females at optimal ages for superovulation
- Collection of embryos after superovulation should be performed as soon as possible, ideally at 0.5 dpc and followed by incubation, in C57BL/6J, GMM at a B6129 and FVB/N backgrounds. Maintenance of embryos up to the 2-cells stage in the female reproductive tract resulted in embryo loss;
- Embryo transfer in C57BL/6J resulted in optimal success rates when 6 embryos were used unilaterally. B6129F1 higher success rates were achieved when 6 embryos bilateral transfers were used but the higher number of pups was obtained using 12 embryos unilaterally;
- Embryo transfer should always consider a minimum number of embryos using unilateral transfers as a priority choice. Bilateral transfers seem to require a minimum number of embryos in B6 to be successful;
- Nevertheless, oocyte or embryo quality was not evaluated in the superovulation nor at embryo collection experiments and, this parameter, could affect the final conclusions achieved for these experiments. Oocyte quality may be dependent on female's age and the use of incubation may also be detrimental for embryo quality.

In addition, distinct microbiota profiles were identified in a specific strain of VCMSH2$^{LoxP/LoxP}$ mice:

- VCMSH2$^{LoxP/LoxP}$ presents a distinct microbiota in two separate areas of the facility; the microbial community revealed differences in alpha-diversity and a shift in the predominant phylum in each area (Firmicutes and Bacteroidetes);
- Differences in the overall bacterial community were also present when beta-diversity was evaluated, with a differentiation of mice by the two distinct areas;
- The number of animals with tumor lesions and their location was not statistically different but differences were found regarding lactoferrin levels, CD8^{+}T cells infiltration and also in inflammatory scores that were distinct in the two zones.

Nevertheless, these variables were not significantly different between animals that develop tumor and animals that did not develop;

- Microbiota characterization should be considered an important hallmark and a valuable strategy to reduce experimental variability and improve reproducibility of animal experiments.

All together, the evidences raised by the current investigation contribute for substantial animal reduction, optimization of different ART techniques and raise awareness regarding the GM impact in mouse disease models.

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