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Original article

Staphylococcus epidermidis is involved in a mechanism for female reproduction in mice

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ABSTRACT

Both external and internal surfaces of organs (e.g., skin, mouth, gut, and intestine) are covered with bacteria, which often contribute to physiological events in host animals. Despite externally opened organs, the presence of bacteria in the mammalian female reproductive tract is uncertain. Here we assessed this problem using wild-type strains of mice, C57BL/6N and ICR. We first demonstrated that bacterial colonies were formed from the oviductal fluid in the C57BL/6N mice with birth experience ("parous"), but not in the mice without birth experience ("non-parous"). Sequence analysis of 16S ribosomal RNA (rRNA) revealed that *Staphylococcus epidermidis* existed in the oviductal fluid of the parous mice, confirmed by immunohistochemical analysis. Furthermore, extinction of bacterial population with intraperitoneal injection of antibiotics, penicillin G and streptomycin, disturbed the regularly implanted pattern of embryos in ICR mice. Our results indicate that symbiotic *S. epidermidis* plays a role in interaction between embryo and uterus upon implantation in mice.

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1. Introduction

Bacteria are inhabited on the external surface and in the lumen of organs in host organisms [1,2]. Based on cooperative relations between bacterium and host organism, the bacteria are provided with a living place, a supply of food, and a constant condition of temperature and humidity from host animals [3,4]. They are often harmless, but also compete with pathogenic bacteria, and protect against diseases in host organisms [5]. The host and the bacteria both gain benefits from this peaceful relation, termed mutualism, a type of symbiosis [6]. As a mutualistic case in humans, the intestine is known to harbor complex societies of bacteria in the lumen [7]. The intestinal bacteria provide us with vitamin B and vitamin K, and help us digest foods [7]. And also, microbial symbiosis factors, such as polysaccharide A (PSA), prevent intestinal inflammatory diseases [8].

In a vagina as a gate of the female reproductive tract, an anerobic lactobacillus, *Doderleïn bacilli*, forms biofilm, a complex aggregation of microorganisms on the mucosa in humans [9]. Its symbiosis has a beneficial effect by inhibiting growth, adhesion or spread of other microorganisms [10]. In the vagina, this bacterium is also known to continually convert glycogen into lactic acid, and prevents pathogens from entering inside the vagina by keeping pH in the cavity acidic [9]. On the other hand, administration of antibiotics and spermicides, a contraceptive substance that stop sperm from moving, to women disturbs such a beneficial relation between host and bacterium [11]. Since *D. bacilli* plays a role in suppressing the pathogen entry, the maintenance of its symbiosis is an important issue for pregnant ability of women.

In mammals, the semen enters to the female reproductive tract along with ejaculated sperm [12]. The semen is known to include immunosuppressive materials potent for protecting ejaculated sperm from attack of female immune system, and weaken the female defensive ability against bacterial entry [13], implying that bacteria may enter the uterus and the oviduct along with the sperm entry. However, symbiotic bacteria have not ever found to enter the female reproductive tract beyond the vagina in mammals, and even more serve in reproductive events of females. Whether the female

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reproductive tract needs the symbiotic relationship between bacterium and reproductive tract is still unclear in mammals including humans. In the present study, we focused on the microbial environment as one of factors regulating the litter size in mice.

2. Materials and methods

2.1. Antibodies and chemicals

A mouse monoclonal antibody (mAb) and a rabbit biotinylated polyclonal antibody (polyAb) against whole soluble and structural antigens of *Staphylococcus epidermidis* and *Staphylococcus aureus* were purchased from Pierce Biotechnology Inc. and abcam Plc., respectively. For immunohistochemical analysis, Alexa647-conjugated IgG and Alexa488-conjugated streptavidin (Life Technologies Corp.) were used. The nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) (WAKO Pure Chemical Industries, Ltd).

2.2. Animals

C57BL/6N and ICR mice (8–12 weeks old) were purchased from Japan SLC Inc., Shizuoka, Japan. Male 8- to 12-week-old B6C3F1 mice, a cross between female C57BL/6N and male C3H mice, were purchased from SLC Inc. (Shizuoka, Japan).

Mice were housed in specific pathogen-free controlled conditions. Food and water were available *ad libitum*. The procedures for performing animal experiments were in accordance with principles and guidelines of the *Care and Use of Laboratory Animals at National Institute for Child Health and Development*.

2.3. Colony formation assay

For colony formation assay, the fluids were collected from the inner cavities of the female and male reproductive organs. One month after C57BL/6N female mice were mated with the male mice and produced their offspring, these female mice were superovulated. Female 8- to 12-week-old C57BL/6N mice were injected with 5 unites of pregnant mare's serum gonadotropin (PMSG; Merck4Biosciences, Darmstadt, Germany). Later, 5 units of human chorionic gonadotropin (hCG; Merck4Biosciences, Darmstadt) was injected. The oocytes were aseptically isolated from oviduct 14–16 h after hCG injection and gently dipped in 70% ethanol. Then, the oviductal fluids including oocyte–cumulus complexes (COCs) were collected from inner cavities of the oviducts. The oviductal fluids were placed in saline solution (Otsuka Pharmaceutical Co., Ltd.) without antibiotics, and spread on MRS agar plates (Kanto Chemical Co. Inc.), which were placed upside down and incubated at 37 °C for 16 h. Concomitantly, three kinds of male reproductive organs, epididymides, seminal vesicles, and preputial glands, were isolated aseptically from 8- to 12-weeks-old C57BL/6N mice. Similarly, after being collected, their inner fluids were seeded on MRS agar plates.

2.4. Polymerase chain reaction (PCR)

To identify the bacterial species, the colonies were picked up with toothpicks, grown in 10 ml liquid MRS medium without antibiotics, and the tubes were incubated overnight at 37 °C on a tube shaker (Thermo Scientific. Inc.). After they were centrifuged, its pellets were subjected to DNA extraction with MidiPrep kit (QIAGEN) and PCR analysis of microbial 16S rRNA genes. Since 16S rRNA gene contains hypervariable species-specific regions, the PCR analysis of this gene can provide signature sequences useful for bacterial identification [14]. The species-specificity of the 16S rRNA genes in microbial colonies was detected by PCR analysis using the following set of primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′, nucleotide no. 8–27 [GenBank Accession no. NR_041920]) and 1525R (5′-AAGGAGGTGATCCAGCC-3’, nucleotide no. 1525–1541 [GenBank Accession no. NR_041920]), and this primer set yielded a band of 1499 bp. The amplified DNA fragments were inserted into pGEM-T Easy vector (Life Technologies Corp.), amplified, and sequenced with ABI3730 capillary sequencer (Life Technologies Corp.) using BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corp.).

2.5. Gram's staining

For immunohistochemical analysis, female reproductive tract and male reproductive organs of C57BL/6N mice were stained with Gram's method, as described previously [15]. To avoid the bacterial contamination and grow, the female reproductive tract and male reproductive organs were fixed with 20% formalin solution, embedded in paraffin, sectioned, stained with Gram's method, and observed under a microscope (BX51; Olympus Corp.).

2.6. Immunohistochemical analysis

Bacteria identified in female reproductive tract and male reproductive organs of C57BL/6N mice were subjected to immunohistochemical analysis. Each tissue of female reproductive tract and male reproductive organs was aseptically collected from females and males, and investigated for the endogenous bacteria. The immunohistochemical analysis was performed using paraffin section of the tissues to find the habitats of two species, *S. epidermidis* and *S. aureus*.

2.7. In vitro fertilization (IVF)

Sperm were collected from the epididymis of 8- to 12-week-old B6C3F1 male mice and induced to capacitate by incubation of them in TYH medium for 90 min in an atmosphere of 5% CO_2 in air at 37 °C before insemination. The final concentration of sperm added to the oocytes was 1.5×10^5 sperm/ml. Oocytes were collected from oviducts of C57BL/6N female mice 14–16 h after injection with 5 units of human chorionic gonadotropin (hCG; Merk4Biosciences, Darmstadt, Germany) into pregnant mare's serum gonadotropin (PMSG; Merk4Biosciences, Darmstadt, Germany)-treated mice. Then, the oocytes were placed in a 30-μl drop of TYH medium covered with paraffin oil (Nacalai Tesque, Inc) equilibrated with 5% CO_2 in air at 37 °C. To investigate the physiological roles of bacteria in the female reproductive functions, IVF was then conducted in fertilization media including different concentrations of each antibiotic, penicillin G and streptomycin (0, 50, or 250 units/ml).

After being fertilized with sperm, the oocytes were transferred to a 30-μl drop of EmbryoMax KSOM medium (EMD Millipore Corp.) including each of antibiotics, penicillin G and streptomycin, KCl, and $MgSO_4 \cdot 7H_2O$, and continuously developed to the blastocyst stage. The rates of two-cell, morula, and blastocyst stages were determined under a differential interference microscope (BX51; Olympus Corp.).

2.8. Determination of litter size and number of implantation sites

Before mating, each of 8- to 12-week-old female and male ICR mice was intraperitoneally injected of 15 units of penicillin G (Sigma–Aldrich, Co. LLC.) and 68.4 units of streptomycin (Sigma–Aldrich, Co. LLC.) every day for 4 days. After treatment, the antibiotic-treated mice were mated with the untreated counterparts. To determine the litter size, the number of pups delivered

from female mice was recorded after mating by placing male mice in the cage. Concomitantly, antibiotic-untreated female and male mice were intercrossed.

For counting the number of implantation sites, 0.1 ml of 1% Chicago blue B suspended in saline solution was intravenously injected to antibiotic-treated and −untreated female mice. Five min after injection, they were sacrificed, and their uteri were isolated, as described previously [16]. The number of blue bands in the uterine was counted as the implantation sites.

3. Results

3.1. Possible effect of environmental factors in litter size

To study the mechanism of sperm-oocyte fusion, we maintained the colonies of *Cd9*$^{-/-}$ mice in the “conventional” animal room in 1998 and 1999 [17], secondly in the “specific pathogen-free (SPF)” animal room in 2003, and thirdly also in the SPF animal room in 2006–2010 [18]. As a control, *Cd9*$^{+/-}$ female mice sustaining the comparable fertility to wild-type female mice were mated with *Cd9*$^{+/-}$ or *Cd9*$^{-/-}$ males. In 1999, *Cd9*$^{+/-}$ embryos at two cell stage were stored in the liquid nitrogen, and they were recovered in the SPF animal room independently in 2003 and 2006, meaning that the genetic background of *Cd9*$^{+/-}$ mice is close among these three periods. However, when the litter size of *Cd9*$^{+/-}$ female mice obtained by us as a control was counted, we found that their sizes significantly differed between conventional and SPF animal rooms, despite the same strain and background (conventional animal room [10.1 ± 0.5 in 1998 and 11.3 ± 0.5 in 1999] *vs*. SPF [6.1 ± 0.6 in 2003 and 6.5 ± 0.4 in 2006–2010]) (Fig. 1). In order to account for this discrepancy, we considered the possibility that environmental factors, including microorganisms, might be involved in reproductive processes in mice.

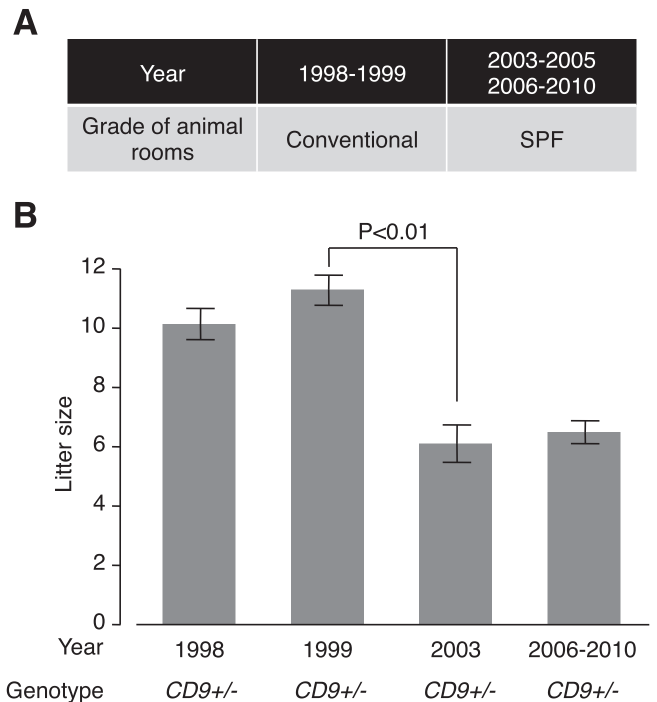


Fig. 1. Litter size of the same strain of mice under different breeding conditions. A, Feeding period of *Cd9*$^{+/-}$ mice under two different conditions against pathogens. To study the mechanism of sperm-oocyte fusion, *Cd9*$^{-/-}$ female mice exhibiting a defect in oocyte fusion with sperm were obtained by crossmating between heterozygote mice in the “conventional” condition in 1998 and 1999, secondly in the “specific pathogen-free” condition in 2003, and thirdly in the “specific pathogen-free” condition in 2006–2010. B, Comparison of the litter size of *Cd9*$^{+/-}$ female mice between two conditions. The *Cd9*$^{+/-}$ embryos at two-cell stage were freeze-stocked in the liquid nitrogen in 1999, and recovered in 2003 and 2006. Values are the mean ± SE.

3.2. The presence of symbiotic microorganisms

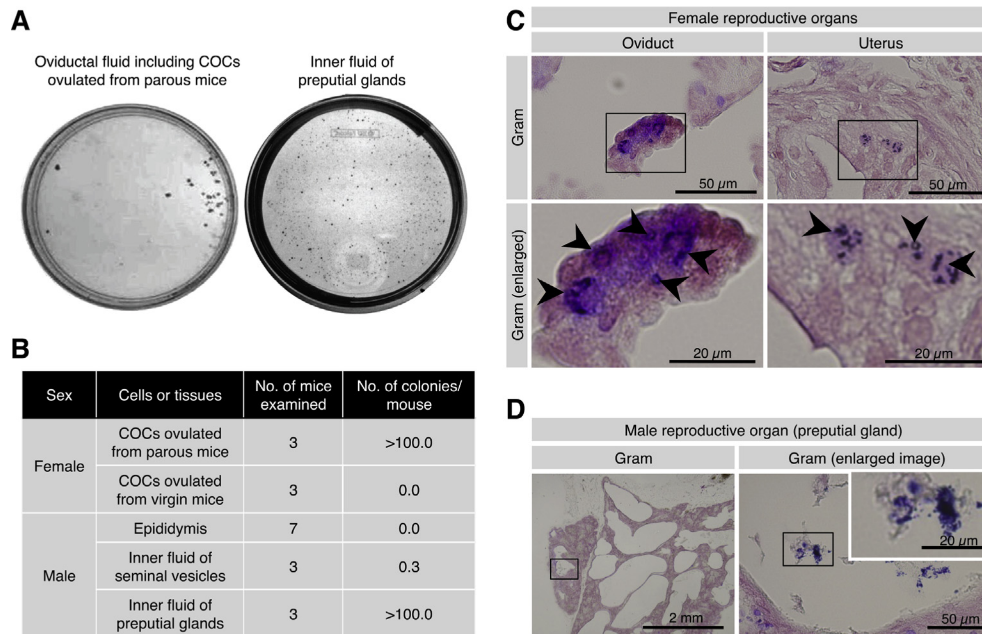
Since the oviduct is a tube that links the ovary to the uterus and an innermost region of the female reproductive tract apart from the vagina [19], accidental contamination of microorganisms is difficult in the oviduct. Given that fertilization occurs in the oviduct [19], we considered that the microorganisms might reach the oviduct along with sperm, grow, and form their colonies in the oviduct. To assess this problem, we first explored whether microorganisms would be inhabited in the oviduct using colony formation assay. Here we used C57BL/6N female mice, which were divided into two groups, the female mice with or without birth experience (hereafter referred to as “parous” or “non-parous”). As depicted in Supplementary Fig. 1, the oviductal fluids including oocyte–cumulus complexes (COCs) were collected from oviducts of superovulated female mice and spread on the MRS agar plates, which were incubated from overnight to 2 days at 37 °C. Concomitantly, the inner fluids of three kinds of male reproductive organs opened externally, “preputial gland”, an exocrine gland located in front of the genitals, “seminal vesicle”, a secretory gland producing semen, and “epididymis”, a sperm-stored elongated organ on the posterior surface of a testis, were subjected to this assay.

After incubation, colonies were formed from the COCs collected from the parous female mice (left image; Fig. 2A). Similarly, colonies were formed from the inner fluids of the preputial glands (right image; Fig. 2A). Quantitative analysis demonstrated that more than 100 colonies were on average formed from the inner fluids including COCs of the parous female mice in triplicate experiments, but not from that of non-parous ones (Fig. 2B). Similarly, more than 100 colonies were on average formed from the inner fluids of preputial glands in triplicate experiments. By contrast, a colony was rarely formed from that of seminal vesicles in triplicate experiments (Fig. 2B). When the colony formation assay was repeatedly (7 times) conducted to the epididymal fluids including sperm, no colonies were detected (Fig. 2B).

To histocytochemically detect the presence of microorganisms in the reproductive tract, we next performed the Gram's staining to sectioned tissues of the oviduct and the uterus of the parous female mice and the preputial gland. To avoid the bacterial contamination and grow, the female reproductive tract and male reproductive organs were fixed with 20% formalin solution, embedded in paraffin, sectioned and stained with Gram's method. As shown in Fig. 2C, Gram-positive areas were detected in the oviduct of the parous female mice (arrowheads in the left set of images; Fig. 2C). In the uterus, the Gram-positive dots were detected inside the macrophages, which were invaded in the uterine tissues, implying that the macrophages may phagocytize Gram-positive bacteria (arrowheads in the right set of images; Fig. 2C). Similarly, parts of lumen of preputial glands were stained with Gram's method (Fig. 2D). These results indicate that Gram-positive bacteria exist inside the oviduct and the uterus of female mice with birth experience and the preputial gland of male mice.

3.3. Identification of symbiotic bacteria

The sequences of 16S rRNA genes were conserved among Gram-positive bacteria, but the species-specific regions have also been identified [20]. The sequencing of 16S rRNA genes has become prevalent in medical microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification [20]. To identify the



Sex	Cells or tissues	No. of mice examined	No. of colonies/ mouse
Female	COCs ovulated from parous mice	3	>100.0
	COCs ovulated from virgin mice	3	0.0
Male	Epididymis	7	0.0
	Inner fluid of seminal vesicles	3	0.3
	Inner fluid of preputial glands	3	>100.0

Fig. 2. Evidences of the presence of symbiotic microorganisms. A, Microbial colonies formed from inner fluids collected from male and female reproductive organs. Female oviductal fluids were collected from the oviducts including cumulus–oocyte complexes (COCs) in the parous and non-parous super-ovulated mice 14–16 h after hCG injection, and subjected to the colony formation assay. Concomitantly, the inner fluids of male reproductive organs, epididymides including sperm, seminal vesicles, and preputial glands, were tested. Microbial colonies grown on MRS agar plates were shown. B, Comparison of the number of colonies formed on the agar plates. C, The oviduct of the parous mice stained with Gram's method. The tissues were fixed in 5% formalin and embedded in paraffin. After being sectioned, the tissues were exposed to 20% formalin to prevent contaminated bacteria from growing on the sectioned tissues. They were then stained with Gram's method. Gram-positive bacteria were stained violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet. Insets in the upper images were enlarged in the lower images. D, The preputial gland stained with Gram's method. Insets in the upper images were enlarged in the lower images.

microbial species habited in the oviducts and the preputial glands, the sequences of 16S rRNA genes were amplified by PCR, and bacterial species were identified by comparison with DNA sequences recorded in databases, as depicted in Fig. 3A. In females, the bacterium isolated from cumulus–oocyte complexes (COCs) was identified as *S. epidermidis* (GenBank Accession no. KF011975) (Fig. 3B). On the other hand, in males, the bacterium isolated from preputial glands was identified as *S. aureus* (GenBank Accession no. KF011976) (Fig. 3B). These staphylococcal species are Gram-positive bacteria, as shown in Fig. 2C and D. Since sperm and semen enter the female reproductive tract upon mating behavior, the *S. aureus* identified in the inner fluids of the preputial glands was predicted to detect in the female reproductive tract; however, this bacterium was not identified at least in the oviduct. This result indicates that distinct bacterial species are inhabited in the inner cavities of reproductive organs of male and female mice.

These findings could also be supported by immunohistological analysis of the oviduct and the preputial gland (Fig. 3C and D). Regarding the immunoreactivity of *S. epidermidis* and *S. aureus*, two antibodies that recognize whole soluble and structural antigens were used, respectively. Immunohistochemical analysis demonstrated that the uterine tissue was reacted with the mAb against the surface antigen of *S. epidermidis* (Fig. 3C). The lumen of the preputial gland was reacted with the mAb against a surface antigen of *S. aureus* (Fig. 3D). These results indicate that *S. epidermidis* and *S. aureus* exist in the female reproductive tract and the male reproductive organ, respectively.

3.4. Influence of treatment with antibiotics in vitro

To study the physiological roles of bacteria in the female reproductive functions, *in vitro* fertilization (IVF) was conducted in TYH medium [21] including different concentrations of antibiotics, penicillin G and streptomycin. The oocytes were collected from superovulated C57BL/6N female mice and the sperm were collected from B6C3F1 male mice. Here, each of these antibiotics was used at the concentration of 0, 50, or 250 units/ml (Supplementary Table 1). As a result, the rates developed to the two-cell stage were comparable among these concentrations of antibiotics (87.5, 84.7, and 80.1%). In contrast, the rates developed to the blastocyst stage were strongly affected (82.5 and 80.0% in the medium including 0 and 50 units/ml, respectively; 0.0% in the medium including 250 units/ml). On the other hand, since penicillin G and streptomycin include 10 μM KCl and 10 μM $MgSO_4 \cdot 7H_2O$, respectively, which are known to affect the developmental ability of mouse embryos, the reduced ability of blastocyst formation may be due to these substances. Therefore, when IVF was performed in the medium including either 10 μM KCl or 10 μM $MgSO_4 \cdot 7H_2O$, the developmental rates were reduced, relative to that without these substances as a control; but the effects were weaker than that of 250 units/ml of antibiotics (Supplementary Table 2 *vs*. Supplementary Table 1B), implying that antibiotic treatment may reduce the developmental ability due to the disappearance of symbiotic bacteria.

3.5. Antibiotic treatment of mice in vivo

To further study the roles of symbiotic bacteria, we first estimated the litter size of female mice treated with antibiotics. As depicted in Fig. 4A, the antibiotics, 150 units of penicillin G and 684 units of streptomycin, were intraperitoneally injected to the ICR female mice every day for 4 days, and mated with untreated male mice. When the litter size was counted, they were comparable between antibiotic-treated and −untreated mice (13.4 ± 0.6 from the antibiotic-treated female mice, N = 11 and 13.3 ± 0.5 from the untreated ones, N = 14) (Fig. 4B).

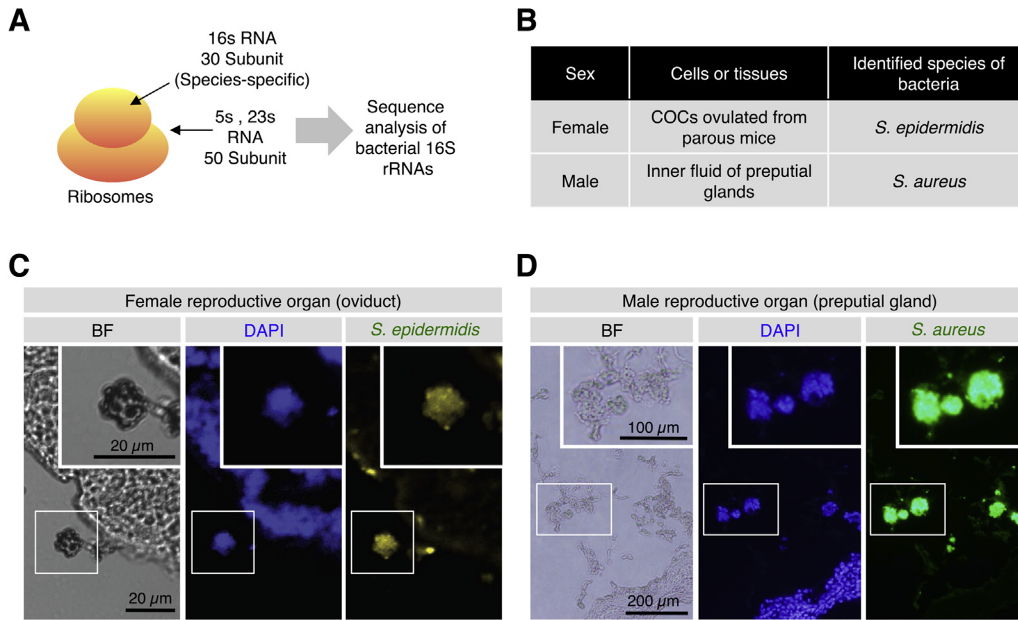


Fig. 3. Identification of microbial species and immunohistochemical analysis. A, Experimental flow exhibiting the identification of microbial species using PCR analysis of 16S rRNA genes. To identify species of colonies, the extracted DNAs were PCR analysis of the microbial 16S rRNA genes. B, A list of bacterial species identified by PCR analysis. *S. epidermidis* was identified in the oviductal fluids of the parous mice, whereas *S. aureus* was in the preputial gland. C, Immunohistochemical analysis of the oviduct of the parous mice. After being sectioned and exposed to 20% formalin, the tissues were immunostained with anti-surface antigen of *S. epidermidis*, and counterstained with DAPI. BF, Bright field. D, Immunohistochemical analysis of the preputial glands. Similarly, the tissues were immunostained with anti-surface antigen of *S. aureus*, and counterstained with DAPI. BF, Bright field.

Next, we assessed the number and the patterns of implantation sites. Five days after plug formation, the implantation sites were monitored with injection of Chicago Blue Sky B, as previously described [16]. When the number of implantation sites per horn in the uterus was counted, they were significantly increased, compared with that of untreated mice (16.1 ± 0.6 from the antibiotic-treated female mice vs. 14.4 ± 0.3 from the untreated ones; P = 0.006) (Fig. 4C), despite no difference between their

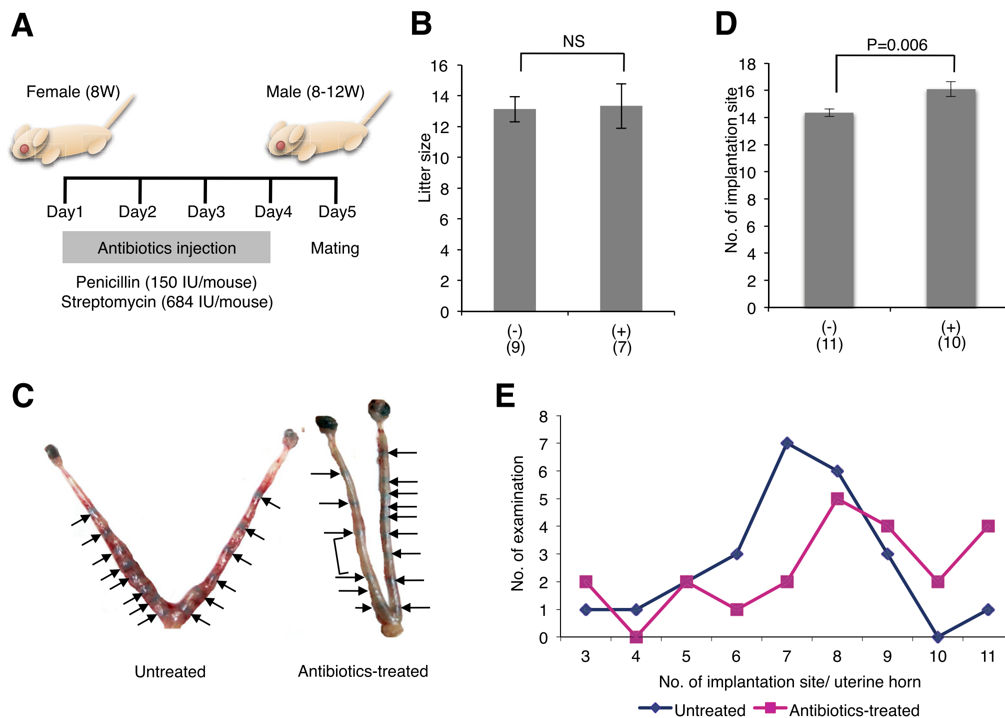


Fig. 4. Disturbed implantation rules in female mice treated with antibiotics. A, Experimental flow of comparison of the litter size between female mice with and without antibiotic treatment. After ICR female mice were treated with antibiotics for 5 days, they were mated with antibiotic-untreated counterparts, and the litter size was counted. As a control, antibiotic-untreated female mice were examined. B, Litter size of female mice treated with or without antibiotics. Values are expressed as mean ± SE. NS, not significant. C, Detection of implantation sites. 5 days after the plugs were formed, the implantation sites were counted using the injection of Chicago Blue Sky B into the tail vein. Right image, antibiotic-treated mice; left image, untreated mice. D, The number of implantation site female mice treated with or without antibiotics. Values are expressed as mean ± SE. NS, not significant. E, Comparison of implantation sites. Values are expressed as mean ± SE.

values (Fig. 4D). Moreover, the patterns of implantation sites were affected. In other words, the implantation sites per a horn of uterus were distributed from 3 to 11 in the antibiotic-treated females, whereas the peak of number of implantation was 7 in the untreated females as a control (Fig. 4E). These results suggest that the disappearance or reduction of symbiotic bacteria disturbs uterus–embryo interaction, leading to the derangement of embryo spacing.

Further, we estimated the litter size of male mice treated with antibiotics. As depicted in Supplementary Fig. 2A, when antibiotics were intraperitoneally injected into male mice every day for 4 days and mated with the untreated female mice. After the antibiotics were intraperitoneally injected into male mice, the lumen of the preputial gland was not reacted with the mAb against a surface antigen of *S. aureus* (lower set of images of supplementary Fig. 3). As a result, the litter size was significantly increased, compared with that of the untreated mice (16.0 $\pm$ 0.8 from the antibiotic-treated mice *vs.* 13.4 $\pm$ 0.6 from the untreated ones; $P < 0.05$) (Supplementary Fig. 2B). In addition, since the litter size of the antibiotic-treated male mice was too much, the pups were often delivered by caesarian section. This result led us to consider that *S. aureus* inhabited in preputial glands may interfere with reproductive functions of mice, and limit the litter size within the normal range. These collected results led to consider that *S. epidermidis* is inhabited in the female reproductive tract after mating, and involved in interaction between embryo and uterus upon implantation; on the other hand, *S. aureus* enters the preputial gland and may compete with the colony formation of *S. epidermidis* in the female reproductive tract. Moreover, the existence of these two Staphylococcal species encouraged us to suppose that bacterial balance may impact the mouse reproduction, and the imbalance may fluctuate the litter size.

4. Discussion

Symbiosis is a close ecological relationship between individuals of different species, and sometimes the symbiotic relationship benefits both species [6]. Our present results indicate that *S. epidermidis* is involved in interaction between embryo and uterus upon implantation. This is demonstrated by the fact that treatment with antibiotics increased the number of implanted sites rather than litter size and disturbed implantation pattern (Fig. 4).

Symbiotic bacteria are known to inhibit pathogen colonization [6]; however, complex host–microbe and microbe–microbe interactions have made it difficult to gain a detailed understanding of the mechanisms involved in the inhibition of colonization. Among symbiotic bacteria, *S. epidermidis* is known to be a major constituent of the normal microbe on healthy human skin, and act as a barrier against colonization of potentially pathogenic microbes [5]. Moreover, *S. epidermidis* secrets the serine proteases Esp, and inhibits biofilm formation and nasal colonization by *S. aureus* [5]. The presence of Esp-secreting *S. epidermidis* in the nasal cavities of humans correlates with the absence of *S. aureus* [5]. Moreover, purified Esp inhibits biofilm formation and destroys pre-existing biofilms of *S. aureus*, also confirmed by *in vivo* studies [5]. These findings indicate that Esp hinders *S. aureus* colonization *in vivo* through the bacterial interference. The present study provides the evidence that there is the bacterial interference between *S. epidermidis* and *S. aureus* in the female reproductive tract.

In female reproduction, implantation is a series of processes between embryo and uterus during the initial period of gestation (i.e., embryo position, attachment, and invasion to uterine epithelial layers) [22]. The intrauterine distribution of embryo implantation sites is remarkably constant in mammalian species [22]. In mice, the embryos implant evenly along the uterine horns [16], and disruption of the orderly implanted pattern leads to the reduction of pregnancy rates [23]. Implantation has been generally considered to consist of at least two events: (1) blastocyst alignment equidistantly along the uterus (hereafter referred to as “embryo spacing”), and (2) embryo adhesion and invasion into the uterus [23]. In rats, the administration of relaxin [24], a potent inhibitor of myometrial activity, disrupts the regular intrauterine distribution of the blastocysts before implantation, indicating that intrauterine myometrial activity contributes to proper embryo spacing [25]. Moreover, lysophosphatidic acid (LPA) has a critical role in the establishment of early pregnancy by affecting embryo spacing and subsequent implantation through its receptor, LPA3 [26]. In mice, targeted disruption of *Lpa3* gene resulted in delayed implantation and embryo crowding, which was associated with a dramatically decreased expression of prostaglandin and prostaglandin-endoperoxide synthase 2 [26]. Since prostaglandin regulates the contraction and relaxation of smooth muscle tissue [27], optimal muscular movement seems to be important in embryo spacing. Therefore, it is reasonable to consider that the substance secreted from *S. epidermidis* may stimulate such muscular movement of the urerus.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.reth.2014.12.003.

Author contributions

KM conceived and designed the experiments. CO performed the experiments. KM, CO and NK analyzed the data. KM, MY and AU wrote the main manuscript text and prepared figures. All authors reviewed the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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