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Production of germ-free fast-growing broilers from a commercial line for microbiota studies

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TITLE:

Production of Germ-Free Fast-Growing Broilers from a Commercial Line for Microbiota Studies

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KEYWORDS:

Chicken, Broiler, Germ-free, Microbiota, Ross PM3, Eggs

SUMMARY:

Here we describe a method for the generation of germ-free chicks from eggs of a commercial broiler line, the Ross PM3. This method can be adapted for the generation of germ-free animals from other poultry species.

ABSTRACT:

Studies of the gut microbiota contribution on the host physiology and immunocompetence are facilitated by the availability of germ-free animal models, which are considered as the gold standard. Nesting birds are ideal models for the production of germ-free animals since there is no need to raise their relatives under sterile conditions. Germ-free chickens are mainly generated from specific-pathogen-free (SPF) experimental lines, which are poorly representative of commercial chicken lines. The method proposed here allowed the production of germ-free chickens from the fast growing broiler line Ross PM3, commonly used by the poultry industry. Eggs were quickly collected after laying at a broiler breeder farm. They underwent a strict decontamination process from the collection to the introduction in a sterile egg hatching isolator. The chicks have been hatched and kept in these sterile isolators during the period

necessary to control their sterility. Originally developed for an experimental SPF white leghorn line, the present protocol has been adapted not only to the Ross PM3 broiler line but also to quails. It therefore represents a robust and readily adaptable procedure to other poultry species and nesting birds of economic, biological or ecological relevance.

INTRODUCTION:

There has been a dramatic increase in scientific and popular interest concerning the contribution of the intestinal microbiota to animal health. The microbiota, consisting of bacteria, viruses, fungi, and archaea that inhabit different niches in the animal's intestine, is implicated directly or indirectly in the regulation of inflammatory, infectious and metabolic diseases affecting not only mammalian species but also livestock, such as poultry[1]. Several animal models were developed to better study the contribution of the gut microbiota to health and disease. For example, germ-free and gnotobiotic animals allow the study of the complete absence of microbes or of a known microbiota, respectively, on the physiopathology of infections[2,3]. However, generating and maintaining these animals require specialized techniques and facilities, and the costs, labor, and skills required to maintain them limit their access to many researchers. Indeed, germ-free animals must be monitored regularly for possible contamination using a combination of bacteria cultivation methods, microscopy, serology, gross morphology, and sequencing-based detection techniques. Similar procedures also apply to other species, such as livestock, where animals are generally bigger and require larger facilities for their breeding and maintenance, which may hamper, to some extent, research on the microbiota.

Poultry, more specifically chickens, are the cornerstone of livestock production worldwide, with a flock population that may exceed 40 billion birds per year. It is the most important source of animal protein in the world (http://www.fao.org/poultry-production-products/en/). Moreover, there are no cultural or religious taboos associated to the rearing or consumption of chickens. The poultry gut microbiota is importantly involved in animal's growth, feed conversion ratio, immunity, pathogen resistance, among many other nutritional, physiological or pathological processes[4]. The generation of germ-free chickens is therefore indispensable to underscore the dialogue between the microbiota and its host[4]. Even if microbial communities inhabit the chicken oviduct[5], the content of an egg freshly laid by a healthy hen is mostly free of microorganisms, the eggshell and membranes possessing mechanical barriers to avoid microorganism invasion[4]. In addition, chicks are easily raised in the absence of their relatives, which, unlike mammals, allows the production of germ-free animals without parental rearing under sterile conditions.

The experimental facility "Infectiology of Farm, Model and Wild Animals" (PFIE, UE-1277, Centre INRAE Val de Loire, Nouzilly, France, https://doi.org/10.15454/1.5572352821559333e12) is part of the French National Infrastructure Network EMERG'IN (https://www.emergin.fr/). PFIE has mastered the production of germ-free chickens to perform various experimental studies for more than 40 years[7,8,9,10,11]. These animals were produced from specific pathogen-free (SPF) eggs from a white leghorn laying line raised in closed breeding since the 1970's. Mainly used for microbiological studies[7,8,9], germ-free birds are experiencing a resurgence of interest with questions such as the contribution of the gut microbiota to behavior[13], nutrient utilization[14], immune development[15] and endocrine activity. Even if some studies have been published using

germ-free broiler lines[16], these studies remain underrepresented compared to studies using experimental layer lines. The evolution of scientific questions towards the crosstalk between the microbiota and its host in poultry health and welfare has led us to adapt our historical protocol to produce germ-free broilers of the Ross PM3 line, the world's most utilized broiler chicken line.

PROTOCOL:

Animal care procedures were carried out in accordance with the guidelines set by the European Communities Council Directive (86/609/EEC) and by the French regulations on animal experimentation.

1. Isolator preparation

1.1. Insert the necessary materials into a rigid isolator under positive pressure: 50 mL tubes, 5, 10 and 25 mL plastic pipettes irradiated feed, autoclaved water and sterile sealed plastic containers.

1.2. Fill the transfer germicidal trap with a 2% quaternary ammonium solution.

1.3. Sterilize the isolator three times with formaldehyde vapor by using 60 mL of formalin (24% formaldehyde) added to 30 g of potassium permanganate per cubic meter (m^3). Move the materials inside the isolator between each sterilization to ensure the sterilization of all contact surfaces.

1.4. Set the isolator temperature to 37 °C for at least 2 days before introducing the eggs. Set the hygrometer to 65-70% of relative humidity (RH) on the day of introduction.

2. Egg collection and incubation

2.1. Collect the eggs from farms selected on the basis of a good hatching rate of the laying hens (at least 80% at the peak of egg production) and their good sanitary status (i.e., absence of common poultry pathogens and absence of diseases within the flock). Visually select clean and flawless eggs on the treadmill.

2.2. Immediately decontaminate the egg surface by dipping them for 5 min in a 1.5% peracetic acid solution at room temperature.

2.3. Transport the eggs to the experimental facility using a box decontaminated with formaldehyde vapor.

2.4. Store the eggs for 24 hours at 4 °C. Repeat the decontamination process described in step 2.2, and place them in a hatching incubator (Day 0) for 19 days.

3. Hatching

3.1. On Day 19, verify the fertility, the viability (motility) and the embryo development by candling them using a light egg candler under sterile conditions. Introduce only live-embryonated eggs into the sterile hatching isolators.

3.2. Decontaminate the selected eggs by spraying 1.5% peracetic acid solution for 30 s or until the entire surface of each egg is covered. Eggs will stay in contact with the spray for 16 min and 30 s during the transfer into isolators.

3.3. Transfer the eggs into the sterile hatching isolators and rinse them with sterile demineralized water before placing them in the hatching space.

NOTE: Animals are hatched and reared in sterile isolators. They are fed ad libitum with a commercial diet sterilized by gamma irradiation and watered with autoclaved tap water provided by water dispensers.

4. Bacteriologic status analysis

4.1. One day after hatching, take a fecal sample directly from the rectum of different chicks and pool them in a sterilized glass tube in order to make this sample representative of all animals within a given isolator.

4.2. The fecal samples being more or less liquid, add an equivalent of 1 mL of stool to 9 mL of thioglycolate broth with resazurin. Add the remaining fecal sample to 9 mL of brain heart infusion broth (BHI) and incubate the tubes at 37 °C without shaking for 18 to 48 h. This will allow the growth of a broad range of aerobic, facultative aerobic and nonfastidious anaerobic species.

NOTE: Thioglycolate broth with resazurin is intended for the detection of nonfastidious anaerobic bacteria but it also enables the detection of aerobic bacteria. This medium complies with the European, American, and Japanese pharmacopoeias for sterility testing[18,19,20].

4.3. Visually observe if any modification in the growth media occurs after 18 hours of incubation. After 48 h, take a drop from the BHI fecal-broth media, place it on a glass slide and observe under a microscope (40X magnification) for the absence or presence of bacteria.

4.4. If there is a suspicion on the presence of bacteria, take a sample from BHI culture and seed it onto BHI agar plates. Incubate at 37 °C for 18 to 48 h.

4.5. If colonies are present, carry out high-throughput techniques such as MALDI-TOF mass spectrometry for precise microbial identification.

NOTE: After 72 h, if the bacteriological analyses are negative, the animals are declared germ-free.

REPRESENTATIVE RESULTS:

Six runs of germ-free chicks' generation were conducted with Ross PM3 eggs coming from two different French farms (**Table 1**). A total of 853 eggs were collected and after two decontamination steps and 19 days of incubation, 86.40% were viable. 490 of these viable eggs underwent a third decontamination step and were introduced into various hatching isolators, for an average hatching rate of 79.80%. This represents a hatching rate of 68.94% compared to the initial number of eggs collected.

However, hatching results vary substantially according to the series carried out: from 41.67% to 88.16% of viable chicks compared to the number of eggs collected. These variations were also observed among the different hatches during the same experiment. Since not all isolators have been used for all runs, it is difficult to exclude an isolator-dependent effect. However, this batch effect was directly correlated with the age of the laying hens (**Figure 1**), where eggs coming from older hens were less viable.

The six runs were carried out using four different hatching isolators. After bacteriological control, the animals from 14 of the 16 isolators were confirmed to be germ-free. This corresponds to a success rate of 87.5%. The two remaining isolators were contaminated by a single environmental and nonpathogenic bacteria.

All animals used for the scientific experiments remained germ-free until the end of the studies, for at least three weeks after hatching.

FIGURE AND TABLE LEGENDS:

Figure 1: Effect of the hen's age on hatching rate

The present figure highlights the hatching rates observed based on the age of the laying hen flock, expressed in weeks of laying.

Table 1: Technical results of the different hatching experiments.

The present table highlights the results of the 6 series of germ-free hatching carried out: number of eggs collected, viability of the embryo at 19 days and viable hatched chicks in the various isolators.

DISCUSSION:

Several methods to generate germ-free chickens have been previously described[7,21,22]. Simple methods, such as the one presented here, use different disinfectants to reduce bacterial load in the egg surface and in the isolators. The most commonly used disinfectants are mercuric chloride, quaternary ammonium, iodoform, sodium hypochlorite and chlorine dioxide solutions. The results are often satisfactory. However, despite the accessibility of these methods, few structures can apply the method and raise the animals in large scale, thus making the utilization of germ-free chickens a relatively rare approach, only used to address very specific scientific questions. The methods, materials and equipment described here allow a highly efficient hatching proportion of germ-free broilers, which remain healthy and sterile for at least 3 weeks (the duration of the scientific experiments to which they were produced).

The results show that the adaptation of the protocol to the production of germ-free chicks from eggs collected in commercial poultry farms is successful. Experience with SPF egg production reveals that hatching efficiency depends primarily on the age of the laying hens and on the quality of collected eggs. Both parameters were taken into consideration when farms were chosen and eggs collected. Using the same method, the average hatching rate of germ-free broilers is much better than that obtained in the last production of SPF laying hens at our facility (79% vs 35%), without affecting animal sterility (87% vs 83%). These differences may be associated to the genetic background of the birds (broilers versus layers) as well as to the quality of the eggshell, which is likely to be more fragile in the eggs of the SPF animals, kept in closed breeding for more than 40 years. Moreover, we also show that transportation of more than 2 h (from the farms to the experimental facility) does not affect hatching efficiency and quality.

Although the sterilization process used for hatching isolators and the protocol for egg decontamination were optimized, around 10% of the isolators were not germ-free. In order to understand the origin of the contamination, it is very important to carry out routine sterility control of the hatched isolators before egg introduction.

Concerning the birds' sterility status, we tried to apply methods that allow the growth of aerobic and nonfastidious anaerobic bacteria from fecal samples, thus assuring that we are detecting live, viable bacteria. These methods comply with international pharmacopeias for sterility testing and represent rapid, easy and cost-reduced techniques to be used routinely. However, molecular biology techniques such as 16S rRNA gene sequencing, although not giving information on bacteria viability, can be applied for the confirmation of the presence of noncultivable bacteria. Indeed, a recent study suggested that some bacteria of the maternal oviduct microbiota appear to be transferred to the embryo through the egg white, later constituting most of the embryo gut bacterial population. Moreover, another study suggests that part of the microbial colonizers harbored in early embryos were inherited from maternal hens, and the gut microbial abundance and diversity were later influenced by environmental factors and host genetics during development[23]. However, the results of these studies are based on DNA sequence analysis, where a large number of those bacteria could be dead or not replicable in the egg white (greatly loaded with antimicrobial molecules). Thomas and collaborators[16] performed sterility testing through the assessment of cultivable aerobic and facultative aerobic bacteria through fecal dropping on BHI plates, thus highlighting the efficiency of standard bacteriological methods for germ-free sterility control. Moreover, in the protocol proposed, we used growth monitoring in thioglycolate broth with resazurin to be able to detect the growth of nonfastidious anaerobic bacteria.

Already used for the production of germ-free quails and chickens, the protocol is adaptable to the production of germ-free animals from most nesting birds and offers perspectives for the study of microbiota contribution on the physiology of these animals. Besides the use of this model to investigate host-microbiota mutual interactions in the poultry gut, it could also be useful for applied research. For example, it could be used to assess safety and efficacy of probiotics derived from chicken gut commensal microorganisms to improve animal's health and robustness.

ACKNOWLEDGMENTS:

The authors are grateful to the breeders and the society Boyé accouvage (La Boissière en Gâtine, France) for the supply of fertilized eggs. This study was conducted under the auspices of the research consortium APR-IA "INTEGRITY' (2017-2019), which was funded by the Région Centre Val de Loire, France.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Shang, Y., Kumar, S., Oakley, B., Kim, W.K. Chicken gut microbiota: importance and detection echnology. *Frontiers in Veterinary Science*. **23**, 5-254 (2018).
2. Grover, M., Kashyap, P.C. Germ-free mice as a model to study effect of gut microbiota on host physiology. *Neurogastroenterology & Motility*. **26** (6), 745-8 (2014).
3. Umesaki, Y. Use of gnotobiotic mice to identify and characterize key microbes responsible for the development of the intestinal immune system. *Proceedings of the Japan Academy, Ser. B, Physical and Biological Sciences*. **90** (9), 313-32 (2014).
4. Carrasco, J.M., Casanova, N.A., Fernández, M.E. Microbiota, gut health and chicken productivity: what Is the Connection? *Miyakawa. Microorganisms*. **7** (10), 374 (2019).
5. Lee, S. et al. Characterization of microbial communities in the chicken oviduct and the origin of chicken embryo gut microbiota. *International Journal of Scientific Reports*. **9** (1), 6838 (2019).
6. Hincke, M.T. et al. Dynamics of structural barriers and innate immune components during incubation of the avian egg: critical interplay between autonomous embryonic development and maternal anticipation. *Journal of Innate Immunity*. **11** (2), 111-124 (2019).
7. Le Bars, J. Demonstration of a protocol for obtaining germ-free chickens. *Annals of Veterinary Research.* **7**, 383-96 (1976).
8. Lafont, J.P. et al. Experimental study of some factors limiting 'competitive exclusion' of *Salmonella* in chickens. *Research in Veterinary Science.* **34** (1), 16-20 (1983).
9. Brée, A., Dho, M., Lafont, J.P. Comparative infectivity for axenic and specific-pathogen-free chickens of O2 *Escherichia coli* strains with or without virulence factors. *Avian Diseases*. **33** (1), 134-9 (1989).
10. Dozois, C.M. et al. Bacterial colonization and in vivo expression of F1 (type 1) fimbrial antigens in chickens experimentally infected with pathogenic *Escherichia coli*. *Avian Diseases*. **38** (2), 231-9 (1994).
11. Schouler, C., Taki, A., Chouikha, I., Moulin-Schouleur, M., Gilot, P. A genomic island of an extraintestinal pathogenic *Escherichia coli* strain enables the metabolism of fructooligosaccharides, which improves intestinal colonization. *Journal of Bacteriology*. **191** (1), 388-93 (2009).
12. Porcheron, G., Chanteloup, N., Trotereau, A., Brée, A., Schouler, C. Effect of fructooligosaccharide metabolism on chicken colonization by an extra-intestinal pathogenic *Escherichia coli* strain. *PLoS One*. **7** (4), e35475 (2012).
13. Kraimi, N. et al. Effects of a gut microbiota transfer on emotional reactivity in Japanese

quails (*Coturnix japonica*). *Journal of Experimental Biology*. **222** (2019).
14. Stanley, D. et al. Intestinal microbiota associated with differential feed conversion efficiency in chickens. *Applied microbiology and biotechnology*. **96**, 1361-1369 (2012).
15. Han, Z. et al. Influence of the gut microbiota composition on *Campylobacter jejuni* colonization in chickens. *Infection and Immunity*. **85** (11), e0038017 (2017).
16. Thomas, M. et al. Gut microbial dynamics during conventionalization of germfree chicken. *mSphere.* **4** (2), e00035-19 (2019).
17. Langhout, D.J., Schutte, J.B., de Jong, J., Sloetjes, H., Verstegen, M.W., Tamminga, S. Effect of viscosity on digestion of nutrients in conventional and germ-free chicks. *British Journal of Nutrition.* **83** (5), 533-40 (2000).
18. European Pharmacopoeia (Ph. Eur.): Supplement 6.3 (official in January 2009), Sterility (reference 01/2009:20601).
19. Japanese Pharmacopoeia (JP): The 4.06 Sterility Test as it appeared in the partial revision of the JP 15th edition (made official March 31, 2009, by the Ministry of Health, Labour and Welfare Ministerial Notification No. 190).
20. United States Pharmacopeia (USP): <71> Sterility Tests as presented in Pharmacopeial Forum, Volume 34(6), Interim Revision Announcement No. 6, December 1, 2008, official on May 1, 2009
21. Harrison, G.F. Production of germ-free chicks: a comparison of the hatchability of eggs sterilized externally by different methods. *Laboratory Animals*. **3** (1), 51–59 (1969).
22. Yokota, H., Furuse, M., Okumura, J., Iwao Task, I. A simple method for production and rearing of the germ-free Chick. *J-STAGE home Nihon Chikusan Gakkaiho.* **55** (8) (1984).
23. Ding, J. et al. Inheritance and establishment of gut microbiota in chickens. *Frontiers in Microbiology*. **8**, 1967 (2017).

Figure

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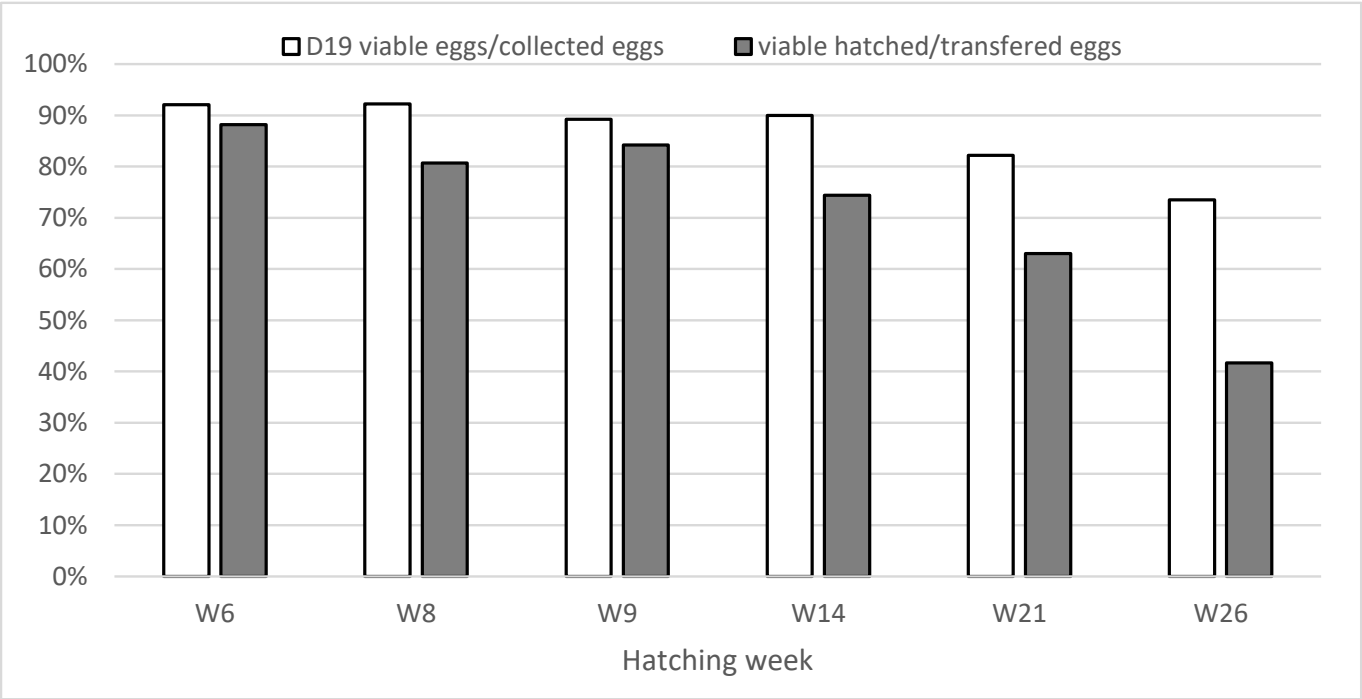

Table1 Click here to access/download;Table;Table1.xlsx

	Collected eggs	D19 viable eggs	**D19 viable eggs/collected eggs**	Eggs tranfered into isolator 1	Eggs tranfered into isolator 2	Eggs tranfered into isolator 3
Run 1	101	93	**92.08%**	23	24	-
Run 2	130	117	**90.00%**	25	25	25
Run 3	132	97	**73.48%**	26	-	26
Run 4	130	116	**89.23%**	36	35	-
Run 5	180	148	**82.22%**	30	30	30
Run 6	180	166	**92.22%**	-	40	40
Total	**853**	**737**	**86.40%**	**140**	**154**	**121**

Eggs tranfered into isolator 4	total of transfered eggs	viable chicks hatched into isolator 1	viable chicks hatched into isolator 2	viable chicks hatched into isolator 3	viable chicks hatched into isolator 4	total of viable hatched chicks
-	47	22	23	-	-	45
	75	20	24	18	-	62
45	97	14	-	13	28*	27
-	71	33	34	-	-	67
30	120	26*	25	17	24	66
-	80	-	35	35	-	70
75	**490**	**89**	**141**	**83**	**24**	**337**

viable hatched/transfered eggs isolator1	viable hatched/transfered eggs isolator2	viable hatched/transfered eggs isolator3	viable hatched/transfered eggs isolator4	**viable hatched/transfered eggs global**
95.65%	95.83%			**95.74%**
80.00%	96.00%	72.00%		**82.67%**
53.85%		50.00%	62.22%	**56.70%**
91.67%	97.14%			**94.37%**
86.67%	83.33%	56.67%	80.00%	**76.67%**
86.67%	87.50%	87.50%		**87.50%**
82.14%	**91.56%**	**68.60%**	**69.33%**	**79.80%**

Name of Material/ Equipment	Company	Catalog Number
2 mL sterile plastic pipettes	Starsted	86.1252.001
50 mL tubes	Falcon	
BHI agar plates	Thermo fisher diagnostic	PO1198A
Brain Heart Infusion broth	Thermo fisher diagnostic	CM1135
Glass tubes with 9 mL BHI broth		
Glass tubes with 9 mL thioglycolate broth with resazurin		
Hatching incubator	Fieme	MG 576
Incubator	Memmert	
Irradiated feed	Safe	U8983G10R
Isolators		
Microbiological safety cabinet	thermon electron corporation	
Microscope Visiscope series 300	VWR	
Pipette aid	Drummond	
Plastic pipettes		
Sterile sealed boxes	Tuperware	
Sterilized glass tube		
Thioglycolate Broth with Resazurin	Merck	90404-500G
Water bath	Fisher scientific	

Comments/Description

home made and sterilized by autoclaving

home made and sterilized by autoclaving

for bacteriological culture, 37 °C
40 kG irradiated
home made. 1 m^3 rigid isolator under positive pressure

model: Hera Safe

diameter
"sovirel"

model: polystat 36, used to incubate 10 min at 100 °C the glass tubes with 9 mL thioglycolate broth with resazurin in order to regenerate the medium

Reference: JoVE61148 "Production of germ-free fast-growing broilers from a commercial line for microbiota studies"

Dear Dr. Steindel,

We appreciate the editorial and reviewers' helpful suggestions, which will certainly improve the quality of our manuscript. Below you will find a point-by-point reply to editorial and reviewers' comments. We hope our manuscript is now suitable for publication and video production.

Editorial comments:

General:

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

We have thoroughly proofread the manuscript.

2. *Please include email addresses for all co-authors in the manuscript itself.*

E-mail addresses were included.

3. *Please include at least 6 key words or phrases.*

We have added 2 more keywords to the revised version.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Pyrex

We removed all commercial language.

Protocol:

1. *Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.*

Line 91: An ethical statement was added to the beginning of the Protocol section.

2. *For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.*

We tried to make all steps clearer.

Specific Protocol steps:

1. 2.1: It's unclear what exactly "on the basis of their hatching rate and health status" means.

Line 109: We have clarified this sentence in the text. To be clearer, we propose the following modification: "Collect the eggs from farms selected on the basis of a successful hatching rate of the

laying hens at least 80 % at the peak of egg production) and their good sanitary status (i.e. absence of common poultry pathogens and absence of diseases within the flock).

Figures/Tables:

1. Table 1: Please upload as a .xls/.xlsx file. Please use periods instead of commas for decimals.

We performed the modifications requested.

References:

1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

2. Please do not abbreviate journal titles.

We have carefully checked each reference

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have provided a Table of Materials in details.

Reviewers' comments:

Reviewer #1:

The manuscript JoVE61148 entitled "Production of germ-free fast-growing broilers from a commercial line for microbiota studies" reports on a procedure to produce germ-free fast-growing broilers from a commercial line. The manuscript deals with a relevant issue in broiler research and production.

Some indications to improve the manuscript are given below.

Specific points:

line 113 Reads: Bacteriologic status analysis. Comment: Why molecular biology techniques (qualitative and quantitative PCR, metagenomic sequencing, etc.) are not used here? They are much more sensitive, precise and powerful than traditional cultivation methods. Is there any official regulation on the procedures to be used to produce germ-free birds?

We understand this reviewer's concern. However, the traditional cultivation methods we used allow the detection of several aerobic and anaerobic bacteria within a few days. For sure, molecular biology techniques are of interest, although they will essentially lead to the determination of the presence (or absence) of bacterial DNA without giving any indication on whether bacteria are viable or not. Nevertheless, we added new information on this topic to the protocol. Please also see the reply to Reviewer #2 for more details.

line 162 Comment: Discussion. This section should be enriched with aspects such as the improvements respect to the previous method, limitations, etc. Also some more information on microbiological aspects would be welcome.

We tried to improve this section as per this reviewer's suggestion.

line 193 Reads: to improve animal's health and Comment: incomplete

line 239:We have clarified this part of the manuscript.

lines: 256-258. Comment: Is not there any more recent reference on this issue?

We preferred to use the seminal references as the techniques utilized in these studies remain relevant to the production of germ-free birds.

Reviewer #2:

What is the difference between the SPF and commercial broilers germfree protocols? Should consider combining the two methods and highly places where procedures should be done differently. For example, commercial broiler eggs are collected from the field and transported to the lab while SPF eggs are obtained locally. Are there more differences?

The differences mainly rely on the rapidity and efficiency of the disinfection and transportation techniques while recovering eggs from environments other than a BSL2 hatching room. We believe we made clear that these steps are the main (and crucial) differences between the two protocols.

Lines 60-62. No proof that their chickens are germ free (see a detailed comment below)

Please see detailed information on the reply to Reviewer #2.

Line 81: Should specify the isolator model used and also described the basic characteristics of the isolator: is it negative pressure, etc

Characteristics were added. However, we are not allowed to cite the isolator model as per Journal's instructions (i.e. "avoid commercial language").

Line 87: Should clarify in the protocol why are the materials being moved.

Line 103: We have now clarified this information.

Line 89: spelling: hygrometer

Corrected.

Lines 93-94: suggested correction:"...egg surface by dipping them in a solution of peracetic acid (1.5% Divosan Plus VT53, JohnsonDiversey, France) for five minutes at room…"

Line 113:Corrected. Thank you for the suggestion.

Lines 97-99: Store the eggs for 24 hours at 4°C, repeat the decontamination process described in step 2.2, and place them in a hatching incubator (Day 0) for 19 days. [no need to restate the hatching incubator conditions - they are stated in step 1.4]

Line117: Modified as per this reviewer's suggestion.

Line 103: the word "different" is not necessary

Line 118: Modified as per this reviewer's suggestion.

Lines 104-105: spraying "for 30 seconds" is not a clear instruction. Consider using a different kind of description, for example, spraying until the entire surface of each egg is covered with disinfectant.

Line123-125: Modified as per this reviewer's suggestion.

Lines 114-119: replace the word "stool" with "feces" or "fecal".

Line 134-136: Modified as per this reviewer's suggestion.

Line 130: This reviewer disagrees with this statement because: 1) Only aerobic bacteria are tested. Besides, only a small percentage of bacteria are culturable.

We partly disagree with this reviewer's comment. Thioglycollate broth with resazurin is intended for the detection of anaerobic bacteria but it also enables the detection of aerobic bacteria (BREWER J.M. - Clear liquid mediums for the « aerobic » cultivation of anaerobes - J.A.M.A. , 1940, Vol. 115, p. 598-600). The broth contains a mixture of peptones, which encourage the growth of most bacteria. The reducing agents (L-cystine and thioglycolic acid) and the yeast extract included in the medium favour the growth of anaerobic bacteria. The redox indicator (resazurin) reveals the presence of oxygen (pink to mauve colour). However, as growth depends on the requirements of each individual microorganism, it is therefore possible that certain strains, which have specific requirements, (substrate, temperature, incubation conditions etc.), may not grow. Finally, this medium complies with the European, American, and Japanese pharmacopoeias for sterility testing. We now added this information to the protocol.

2) Surface decontamination of eggs does not mean elimination of bacteria. The reviewer disagrees with the authors' argument that "The content of an egg freshly laid by a healthy hen is free of microorganisms, the eggshell and membranes possessing mechanical barriers to avoid microorganism invasion". It is established that eggs are not formed in a sterile reproductive system [see Lee et al, Sci Rep. 2019 May 2;9(1):6838.].

The authors agreed that some microorganisms could be embedded within the egg during oviposition. However, to face bacterial growth that could impair embryo viability, embryonated eggs possess many weapons such as antimicrobial molecules (lysozyme, defensins, ovotransferrin) (Hincke et al., J. Innate Immun. 2018). These molecules may also contribute to limit the growth of certain bacteria derived from the hens and present in the egg white, as shown by Lee et al. (Sci Rep. 2019).

Thus, without treatment with antibiotics during the first few days of life, it might be impossible to raise truly germfree chickens. The authors should address this in the Discussion. This reviewer is aware of the study by Thomas et al (mSphere. 2019 Mar 27;4(2). pii: e00035-19) which similarly declared chickens to be germfree based on the absence of viable bacteria.

In Thomas et al. (mSphere, 2019), the germ-free status of the chicks was only checked through fecal dropping on BHI plates. We agree this technique will only allow the detection of aerobic and facultative aerobe bacteria. This is why we used thioglycolate broth with resazurin to be able to detect the growth of nonfastidious anaerobic bacteria. We added this information to the protocol.

Curiously, that study did not report 16S rRNA gene data from uninoculated "germfree" chickens which obviously should have been included as controls. Hence, while the current work in this study and other published studies are encouraging, culture-independent methods such as 16S rRNA gene sequencing should be the gold standard for sterility confirmation.

Although we agree with the reviewer on the relevance of culture-independent methods, 16S rRNA gene sequencing, for example, will only lead to the determination of the presence (or absence) of bacterial DNA without giving any indication on bacteria viability. Thus, bacterial culture (as a first intention) and culture-independent methods can be used in tandem to address different questions concerning sterility.

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Title of Article:	Production of germ-free fast-growing broilers from a commercial line for microbiota studies
Author(s):	Guitton E., Faurie A., Lavillatte S., Chaumeil T., Gaboriaud P., Bussière FI., Laurent F., Lacroix-Lamandé S., Guabiraba R., Schouler C.

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