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.