

## Microbiota of the sheep's maxillary sinus as an experimental model

Microbiota do seio maxilar da ovelha como modelo experimental

Microbiota del seno maxilar de la oveja como modelo experimental

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### Abstract

**Objective.** The aim of the study is to analyze the microbiota of the maxillary sinus of sheep as a viable biome to carry out experimental studies of biomaterials. **Materials-Methods.** 13 sheep (eight in isolation and five loose created) were used, making 26 maxillary sinuses, where swabs were performed by an extra-oral approach for microbiological analysis. Statistical tests such as Fisher's exact test, Mann Whitney U test, chi-square test, and proportional means were used in the experiment. **Results.** Only eight maxillary sinuses had positive bacterial growth. The presence of *Staphylococcus aureus* was statistically significant relative to other microorganisms found. On the other hand, among the animals in isolation and those raised loose, there was no statistically significant difference in the presence of *Staphylococcus aureus*. **Conclusion.** *Staphylococcus aureus* is the predominant bacteria in the maxillary sinus of this experimental model. The difference in environment and feeding did not influence the microbiota of the maxillary sinus.

**Keywords:** Maxillary sinus; Microbiota; Sheep; Experimental model.

### Resumo

**Objetivo.** O objetivo do estudo é analisar a microbiota do seio maxilar de ovelhas como um bioma viável para realizar estudos experimentais de biomateriais. **Materiais-Métodos.** Foram utilizadas 13 ovelhas (oito isoladas e cinco criadas soltas), fazendo 26 seios maxilares, onde foram realizados esfregaços através de uma abordagem extra-oral para análise microbiológica. Foram utilizados os testes estatísticos exato de Fisher, Mann Whitney U, qui-quadrado, e meios proporcionais. **Resultados.** Apenas oito seios maxilares tiveram um crescimento bacteriano positivo. A

presença de *Staphylococcus aureus* foi estatisticamente significativa em relação a outros microrganismos encontrados. Por outro lado, entre os animais em isolamento e os que foram criados soltos, não houve diferença estatisticamente significativa na presença de *Staphylococcus aureus*. **Conclusão.** O *Staphylococcus aureus* é a bactéria predominante no seio maxilar deste modelo experimental. A diferença no ambiente e na alimentação não influenciou a microbiota do seio maxilar.

**Palavras-chave:** Seio maxilar; Microbiota; Ovelha; Modelo experimental.

### Resumen

**Objetivo.** El objetivo del estudio es analizar la microbiota del seno maxilar de las ovejas como bioma viable para realizar estudios experimentales de biomateriales. **Materiales-Métodos.** Se utilizaron 13 ovejas (ocho aisladas y cinco sueltas creadas), lo que supone 26 senos maxilares, en los que se realizaron hisopos por vía extraoral para su análisis microbiológico. En el experimento se utilizaron pruebas estadísticas como el test exacto de Fisher, el test U de Mann Whitney, el test de chi-cuadrado y las medias proporcionales. **Resultados.** Sólo ocho senos maxilares presentaron un crecimiento bacteriano positivo. La presencia de *Staphylococcus aureus* fue estadísticamente significativa en relación con otros microorganismos encontrados. Por otra parte, entre los animales aislados y los criados sueltos, no hubo diferencias estadísticamente significativas en la presencia de *Staphylococcus aureus*. **Conclusión.** *Staphylococcus aureus* es la bacteria predominante en el seno maxilar de este modelo experimental. La diferencia de entorno y alimentación no influyó en la microbiota del seno maxilar.

**Palabras clave:** Seno maxilar; Microbiota; Oveja; Modelo experimental.

## 1. Introduction

The maxillary sinus is a pneumatic cavity in the jaw bone, with a single output (middle nasal meatus) that communicates with the nasal cavity. The maxillary sinuses give a lighter weight to bones and act as a resonance chamber during the speech. Its walls are lined up by a respiratory mucosa with ciliated cylindrical pseudostratified epithelium that promotes the cleaning of the sinus.

The maxillary sinus microbiota has been studied either in healthy sinuses (Jiang, *et al.*, 1999; Brook, *et al.*, 1981; Posawetz, *et al.*, 1991) or sick ones (Posawetz, *et al.*, 1991; Su, *et al.*, 1983) for the understanding of infectious pathologies that often affect them. Its content is a controversial element because of the way for obtaining the analysis.

For healthy maxillary sinuses, it has been said that they did not have bacteria inside (Su, *et al.*, 1983), however, others claim that the maxillary sinus is not sterile, presenting positive bacterial growth (Jiang, *et al.*, 1999; Timmenga, *et al.*, 2003) or with all sinuses having bacteria (Brook, *et al.*, 1981). Nevertheless, all studies were done nasally (middle nasal meatus, endoscopy) (Timmenga, *et al.*, 2003; Ramakrishnan, *et al.*, 2017; Koutsourelakis, *et al.*, 2018) or by mouth (Morawska-Kochman, *et al.*, 2017), both of them with high possibility of contamination.

This paper proposes an analysis of the maxillary sinus microbiota through an extra-oral approach eliminating the possibility of contamination by microorganisms of the nasal and oral cavities.

## 2. Methodology

This is an unprecedented experimental study in which sheep were used as an experimental model because the evaluated structure (maxillary sinus) has many similar characteristics to humans, such as cortical bone thickness (Alsafy *et al.*, 2021; Masoudifard *et al.*, 2022). The microbiological analyses were based on the possible microbiota found in the upper airways and performed at the microbiology department of the Federal University of Sergipe, following all the criteria and care for the proper development of the microorganisms collected, as described below in the Microbiological Analysis topic. The surgical procedure was performed by a team of oral and maxillofacial surgeons respecting all the basic precepts of surgery.

This work was approved and supervised by CEPAP (Ethics Committee for Production Animals) of the Federal University of Sergipe (no. 08/12) in accordance with CONCEA (National Council for Animal Experimentation Control). This study conformed with the ARRIVE Guidelines. The choice of animal model was based on the best biological response found by the species compared to other study models (Smith *et al.*, 2018; Hudaifa *et al.*, 2022). Eight female sheep, with an average

age of 1 year and an approximate weight of 45 kg, were provided by the Federal Institute of Sergipe, which presents a breeding facility for research and teaching (accompanied by veterinary professors from the institution), where the surgeries were also performed. The animals were isolated in covered stalls and suspended 1 meter from the ground, previously disinfected with calcium oxide and chlorine, two weeks before the installation of the animals that were fed with hay and pelleted feed, free freshwater for consumption, with natural day/night cycle. After a four-week adaptation period, the animals were submitted to a surgical procedure. In the experiment, in addition to these, five more sheep that lived free in the pasture in a region distinct from the isolated animals. No animals were euthanized to continue the study and the postoperative period was performed with surgical wound care and analgesic and antibiotic medication.

### **Surgical Procedure**

The animals were subjected to fasting and trichotomy 24 hours before surgery. Atropine (0.02 mg/kg) was used subcutaneously 15 minutes prior to anesthesia induction with xylazine 2% (20 mg/ml) at 0.2 mg/kg by deep intramuscular via associated with ketamine (100 mg/ml) in a dose of 8 mg/kg. After induction, antisepsis was carried out with topical iodine 10%, sterile drapes, and a block with local anesthetic (lidocaine 2% with vessel constrictor) was provided in the area of incision, which was made in the infraorbital rim portion with a craniocaudal flap in the lateral region of the snout (Figure 1). The tissue was dissected by planes and the periosteum separated from the bone to expose the anterior wall of the maxillary sinus.

**Figure 1** - Extra-oral approach approaching the nose and the eyelid's infra-eyelid region.



Source: Authors.

The figure above of the extra-oral access demonstrates the design of the flap and the anatomical references. This access was necessary to avoid contamination of the swab if it were taken orally or nasally.

An opening was made with a surgical drill in the anterior wall of the maxillary sinus. With similar approach to that used by Perini et al. (2020). After opening, the samples were collected with sterilized swabs containing Stuart Transport Medium. A swab was inserted into the maxillary sinus, which was rubbed throughout the mucosa and maintained in the region for over a minute to better absorb the mucus (Figure 2). The swab was removed and immediately immersed in Stuart medium and sealed for analysis. After the swab was performed, an implant by maxillary sinus was installed in the sheep kept in isolation, transfixing the Schneiderian membrane to expose the turns of the implants to the sinus antrum and to evaluate the alteration of the sinus microbiota three months after the first surgery (second research stage) and seven years after the first surgery (third research stage, with results being analyzed).

**Figure 2** - Swab being performed inside the maxillary sinus extra-oral approach.



Source: Authors.

The figure above demonstrates the insertion of the swab into the maxillary sinus extra-orally without touching the animal's skin, thus avoiding cross-contamination of the culture.

### **Microbiological Analysis**

The swabs were transported to the laboratory on an isothermal box and analyzed immediately. Each swab was grooved in triplicate in a suitable culture medium. Blood agar, chocolate agar, Mac Conkey agar, mannitol salt agar and Sabouraud agar were also used. Blood agar plates were incubated in an anaerobic jar. After completion of the grooves, the plates were incubated at 35°C for 24-48h. Sabouraud agar plates were kept at room temperature for seven days. Where there was growth, four colonies were selected from each plate and subjected to morphology and staining investigation, and

biochemical (Rugai) test. After such identification, colonies were chopped in tubes having brain-heart infusion agar and glycerol (BHI) and kept at -12 ° C.

### Statistical Analysis

Statistical tests such as Fisher's exact test, chi-square test, proportional means test, and binomial test were used in the experiment.

### 3. Results

Of the 26 maxillary sinuses analyzed, only eight had positive growth, distributed over six animals – four isolated animals and two loose ones. This difference between sinuses with positive and negative growth was not statistically significant (Binomial test) (Figure 3). Among the four individual animals, two presented bilateral positive growth (Table 1).

**Table 1** – Microbial growth in confined sheep.

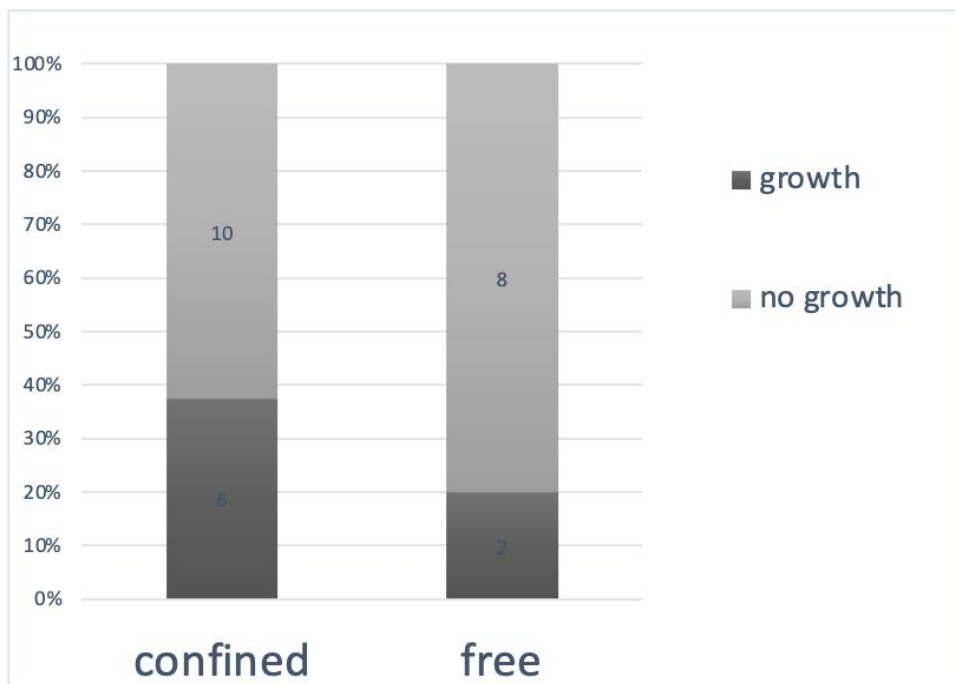
Sheep	01	02	03	04	05	06	07	08
Right sinus	X	X	X	X	X	B	A	D
Left sinus	X	X	X	A	X	A, C	X	E

A - *Staphylococcus aureus*, B - *Staphylococcus epidermidis*, C - *Bacillus sp*, D - *Haemophilus somnus*, E – yeasts, X – no growth.

Source: Authors.

The table above shows the distribution of microorganisms in the maxillary sinuses with a description of the species found.

**Figure 3** – Relationship between free and confined sheep for microbial growth in the maxillary sinus.



Source: Authors.

In the figure above it can be seen that, in both groups, there was no difference between the breasts of the free and the confined ewes as well as between the positive and negative growth of microorganisms.

Among the microorganisms isolated, the following were identified: *Staphylococcus aureus*; *Staphylococcus epidermidis*; *Bacillus sp*; *Haemophilus somnus* and yeasts. There was a statistically significant difference of 5% between the presence of *Staphylococcus aureus* and *Staphylococcus epidermidis* (proportional means test) and statistically significant difference of 1% between *Staphylococcus aureus* and other microorganisms (proportional means test).

Among the animals raised loose, two of them had only one maxillary sinus with positive growth (Table 2). None of the animals had accumulation of mucus and all mucous membranes had healthy aspect in direct view. There was no statistical difference between the groups (confined and loose) about the amount of maxillary sinus with bacterial growth (Fisher test,  $p = 0.312$ ,  $p > 0.05$ ). There was no statistically significant difference in the presence of *Staphylococcus aureus* between confined or loose animals (chi-square test,  $p = 0.747$ ,  $p > 0.05$ ).

**Table 2** – Microbial growth in free sheep.

Sheeps	09	10	11	12	13
Right sinus	X	A	X	X	X
Left sinus	X	X	X	B	X

A - *Staphylococcus aureus*, B - *Staphylococcus epidermidis*, X – no growth.

Source: Authors.

Table 2 shows the distribution of microorganisms detected in the group of free-ranging animals. According to the analysis it is possible to verify that most of the sinuses did not show microbiological growth and there was no difference between the right and left maxillary sinuses.

#### 4. Discussion

The choice of animal was based on the good response to biomaterials (Sirak et al. (2018); Iezzi et al. (2021); Sheftel et al. (2019); Mastrangelo et al. (2019)), its anatomical proximity to the cortex, and volumes similar to those of homes as described by Alsafy et al. (2021) and later by Masoudifard et al. (2022) in their tomographic analysis giving a detailed description of the anatomy of the head of this experimental model. The results of this study show that the maxillary sinus is not a sterile environment, which is according to Jiang *et al.* (1999) that found a variety of positive and negative gram, aerobic and anaerobic bacteria, however, 48.4% of the swabs had negative growth in the maxillary sinuses examined and considered normal. The chances of finding microorganisms inside, in an analysis, are equal to the chances of not finding them, probably due to their self-cleaning ciliary capacity.

The work of Jiang *et al.* (1999) does not report the distribution of those bacteria in the sinuses, whether the culture was mono or multi-bacterial. In our study, we had most of the sinuses without growth (69%) which was statistically significant. Of the eight sinuses with positive growth, only one sinus had two bacterial species, and others had mono-bacterial growth. In the same way that the analysis of the microbiota made by Timmenga *et al.* (2003), in 17 patients without clinical signs of sinusitis, showed that 11 out of 17 maxillary sinuses had negative cultures (65%), there was poly-bacterial growth in two cultures, while just one bacterial species was found in the other four sinuses.

Morawska-Kochman *et al.* (2017) demonstrate, by scanning electron microscopy of the maxillary sinus mucosa of healthy patients, the existence of microorganisms in 93% of the analyzed sinuses. The authors found that the bacteria are dispersed or concentrated in single biofilm microcolonies, on the edge of the mucosa that covers the ciliary epithelium. In our study, we found that only 30% of the maxillary sinuses had detectable microorganisms in the swab. This concentration at the edge of the mucosa, demonstrated by Morawska-Kochman *et al.* (2017) may be able to explain the prevalence found in our study.

The arrangement of microorganisms described by Morawska-Kochman *et al.* (2017) can explain the difficulty in capturing microorganisms by the swab, because the cilia of the epithelium project these bacteria to their periphery. The authors also mention that local factors can influence the development of acute and chronic sinusitis such as bacterial, fungal, viral infections, deficiency in mucociliary function, edema in the sinus mucosa and impaired sinus drainage.

In the study by Koutsourelakis *et al.* (2018), the presence of staphylococcus was detected in 100% of the samples, just as in our study, the presence of *Staphylococcus aureus* was significant. It is likely that *Staphylococcus aureus* is indigenous to the maxillary sinus biome. According to the authors, the normal microbiota of the maxillary sinuses is still not well defined and seems to vary widely among individuals. According to Ramakrishnan *et al.* (2017) it is not known whether each maxillary sinus individually has a unique microenvironment and a unique associated microbiota, or whether they are very similar throughout the process. Although small idiosyncratic differences in the microbiome have been observed between anatomical sinonasal subsites, interpersonal variability has largely overcome these differences.

According to Twaruzek *et al.* (2014), factors that incline to fungal sinusitis can also be associated with environmental conditions, such as high concentration of fungal spores in the air of certain workplaces (farmers, gardeners, carpenters), or in wet environments and poorly ventilated rooms. When comparing, in our study, two groups of animals living in different environments (loose in the pasture and isolated and disinfected stalls), we can note that there was no great difference in bacteria found in both groups. On the contrary, the animals loose in the pasture presented a smaller range of bacterial species and a greater proportional number of maxillary sinuses without growth, thereby decreasing the environment influence strength, for this analysis and reinforcing that the bacteria that were found are within the normal range.

There were no anaerobic bacteria in this study. The work done by Brook (1981) found aerobic and anaerobic bacteria in patients subjected to nasal septum correction surgery, where it grows the possibility of already having patients with a subclinical disorder. It is not very logical to find anaerobic bacteria in oxygen-rich environment, except if these bacteria are facultative anaerobic, or if there is an obstruction of the sinus drainage pathway, decreasing the oxygen supply which could characterize a sick maxillary sinus.

Manarey *et al.* (2004), in a retrospective analysis, reported the presence of *Staphylococcus aureus* resistant to methicillin (9.22 %) in chronic sinusitis and that these bacteria represent the true pathogen and not only colonization. Wald (2012) questioned the influence of *Staphylococcus aureus* in acute sinusitis in children and adults, under the maxillary sinus aspiration technique, since the same concentrations of these bacteria were found in healthy and sick patients. We have found *Staphylococcus aureus* in 50% of the sinuses, which showed positive growth, with an endogenous and dominant bacterium in this experimental model. The *Staphylococcus aureus* prevailed in the samples, which still does not mean that it is the causative agent of acute infections. That will be analyzed in future studies.

The technique used for taking samples excludes any possibility of contamination of the nasal and oral passages, bringing greater reliability to the study. Jiang *et al.* (1999) evaluated healthy maxillary sinus through the oral passage, presented the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus viridans* (commonly found in the oropharynx) showed to be 50% of the prevalent bacteria. The authors did not mention if there was maxillary sinus with negative growth. Timmenga *et al.* (2003) evaluated the composition of healthy maxillary sinuses, through the nasal passage,

and showed the *Streptococcus viridans* and anaerobic bacteria. The authors did not mention the presence of *Staphylococcus aureus*, and 65% of sinuses showed negative growth.

Evidently, it is not possible to provide humans with the same sheep access. There is a similar one in humans, but it is to remove tumors in the region where the anatomy is already endangered by the injury, not being considered healthy. Therefore, it is not feasible for any maxillary sinus analysis under normal conditions.

## 5. Conclusion

Based on the results derived from the current study our limitations are restricted to the experimental model used, since it would be unethical and impractical to carry out such a study in humans. Despite such limitations, the results will serve as a reference for future studies investigating the maxillary sinus, whether for cases of infection or for the study of biomaterials.

The present work provides unprecedented evidence that the maxillary sinus of the experimental model used is not a sterile environment, with *Staphylococcus aureus* being the predominant bacterium. The capture of microorganisms in the maxillary sinus mucosa is difficult, because this environment has a cleaning mechanism through its ciliary system. The difference in environment and diet did not influence the microbiota of the maxillary sinus.

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