



TWO ALTERNATIVE METHODS FOR ANAEROBIC CULTIVATION OF *BACTEROIDES FRAGILIS* ATCC 25285 COMPARED TO THE EFFICIENCY OF CULTIVATION IN A GAS PACK SYSTEM

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ABSTRACT

Cultivation of anaerobes is not a routine procedure. In most cases, it is carried out in reference laboratories with the help of special equipment, including an anaerobic chamber or anaerobic jar with Gas pack system. There are some older methods so-called the Candle jar system and the Fortner principle.

The aim of the study is to compare two alternative methods for anaerobic cultivation of *Bacteroides fragilis* ATCC 25285 with cultivation by Gas Pack system, which is a widely used and well studied method.

We made anaerobic cultivation using the Gas Pack system, Candle jar and Fortner principle. The bacteria were seed in two different types of agar media - suitable for anaerobic cultivation Wilkins-Chalgren agar and Mueller-Hinton agar, useful for antibiotic sensitivity testing.

The results of our study showed that in all bacterial cultures, *B. fragilis* ATCC 25285 demonstrated heavy growth. The Candle jar system and Fortner principle were found to be a sensitive and cost-effective alternative that might be used in resource-limited settings.

Keywords: *B. fragilis*, cultivation, Fortner principle, Candle jar, alternative methods,

INTRODUCTION

Bacteroides fragilis are Gram-negative rod-shaped bacteria. Most healthy adult microbiota are dominated by just two bacterial phyla – the Gram-positive Firmicutes (many genera) and the Gram-negative Bacteroidetes (primarily Bacteroides, Alistipes, Parabacteroides, and Prevotella) [1]. At the same time, *Bacteroides* are a common cause of endogenous infections [2] and the commonest anaerobic bacteria associated with clinical infections, particularly those associated with infections of the mucous membranes and adjacent tissues [3, 4]. Bacteroides

are obligate anaerobes, but they are one of the most aerotolerant anaerobes, and according to Baughn and Malamy, 2004 [5], they may actually slowly divide in the presence of nanomolar concentrations of oxygen.

Cultivation of anaerobes is not a routine procedure. In most cases, it is carried out in reference laboratories with the help of special equipment, including an anaerobic chamber. More accessible methods include the use of an anaerobic jar in which a Gas pack system is placed to create an anaerobic environment chemically. Other similar methods are the use of vented anaerobe jars (usually consisting of N₂ 85%, CO₂ 10% and H₂ 5%), anaerobic gassing cannula (supply of stream of anaerobic gas into the culture vessel) [6] and others.

Some older methods of so-called physical and biological anaerobic cultivation are described in the literature. The biological method, which is called the Fortner principle, utilized the ability of rapidly growing facultative anaerobes to reduce the oxygen tension in a closed system, thus making possible the growth of oxygen-sensitive organisms [7, 8] such as *Bacteroides*. The principle includes using an aerobic organism cultivated together with testing anaerobe into one nutrient medium. The aerobic organism multiplies for the first 24 hours using oxygen in the Petri dish. Then the anaerobic bacteria start to grow, and within the next 24 to 48 hours, we may see the macroscopic visible bacterial colonies.

Anaerobic cultivation by physical method includes using a Candle jar. With the help of a lighted candle in the apparatus creates anaerobic conditions, in the combustion process releases carbon dioxide and consumes oxygen. By combustion of a white wax candle in the candle jar system, 4-5 per cent CO₂ is generated with instantaneous lowering of the oxygen level inside the sealed jar, leaving approximately 1-2 per cent un-burnt oxygen [9, 10]. This technique is not so typical for the purpose

of anaerobic cultivation; it's more often used for microaerophiles cultivation, which requires lower levels of oxygen than are present in the atmosphere and an elevated concentration of carbon dioxide. The atmosphere most commonly used for culturing *Bacteroides* contains 5 or 10% CO₂ [6]. At the same time, carbon monoxide can be inhibitory to some anaerobic organisms. However, we decided to investigate *Bacteroides fragilis* to grow successfully in the Candle jar system.

Aim

The aim of the study is to compare the effectiveness of three different methods for anaerobic cultivation of *Bacteroides fragilis* ATCC 25285.

MATERIALS AND METHODS

The study was conducted in a training microbiological laboratory in Medical university-Varna, Bulgaria, in March 2021. We investigated the possibility of culturing *B. fragilis* ATCC 25285, creating anaerobic conditions by three different methods.

Anaerobic cultivation using a Gas Pack system. We performed dense seeds of *B. fragilis* in two different types of agar media - suitable for anaerobic cultivation Wilkins-Chalgren agar and Mueller-Hinton agar, useful for antibiotic sensitivity testing. For the purposes of anaerobic cultivation, we used an anaerobic jar (*Schuetz-biotec "eco"*, Germany) with an Anaerob Pack system (H₂+CO₂) and an indicator with methylene blue for an anaerobic environment. We put the Petri dishes with nutrient media upside down and cultivate them in the incubator for 48 hours at 35°C. The methodology is performed in triplicate.

Anaerobic cultivation using Candle jar. We made dense seeds of *B. fragiles* on Wilkins-Chalgren agar and Mueller-Hinton agar. The glassware was left with a freely moving lid. The procedure was made in triplicate. We put the Petri dishes upside down in a candle jar, and then we placed a lighted candle on them and closed the lid by carefully sliding it on top. After a minute, the candle flame went out. We sealed the peripheral edges of the vessel with melted paraffin and cultivated bacterial cultures at 35°C/48 hours.

Biological method for anaerobic cultivation – Fortner principle. We used Wilkins-Chalgren agar and Mueller-Hinton agar for *Bacteroides* cultivation. With a sterile knife, we cut a strip from the agar, which divided it into two halves. A strain of *Bacteroides fragilis* was streaked onto one half of the medium, and a strain of *Staphylococcus aureus* was streaked onto the other half. Then we turned upside down only the lid of the Petri dish and sealed it together with the bottom with melted paraffin. In this way, we closed the glassware hermetically and stopped the supply of oxygen from the outside. The bacteria cultures were incubated at 35°C for 48 hours. We repeated the experiment, but *S. aureus* was replaced by a strain of *Escherichia coli*. All the procedures were performed in triplicate.

RESULTS AND DISCUSSION

The results of our study showed that in all twenty-four bacterial cultures we performed, *B. fragilis* demonstrated heavy growth. These 24 bacterial seeds were made following three methods for anaerobic cultivation of *Bacteroides fragilis* ATCC 25285: cultivation onto Wilkins-Chalgren agar and Mueller-Hinton agar, using Gas Pack system (6 bacterial seeds); cultivation onto Wilkins-Chalgren agar and Mueller-Hinton agar, using a candle jar (6 bacterial seeds); cultivation onto Wilkins-Chalgren agar and Mueller-Hinton agar, using Fortner principle with strains of *Staphylococcus aureus* and *Escherichia coli* (12 bacterial seeds).

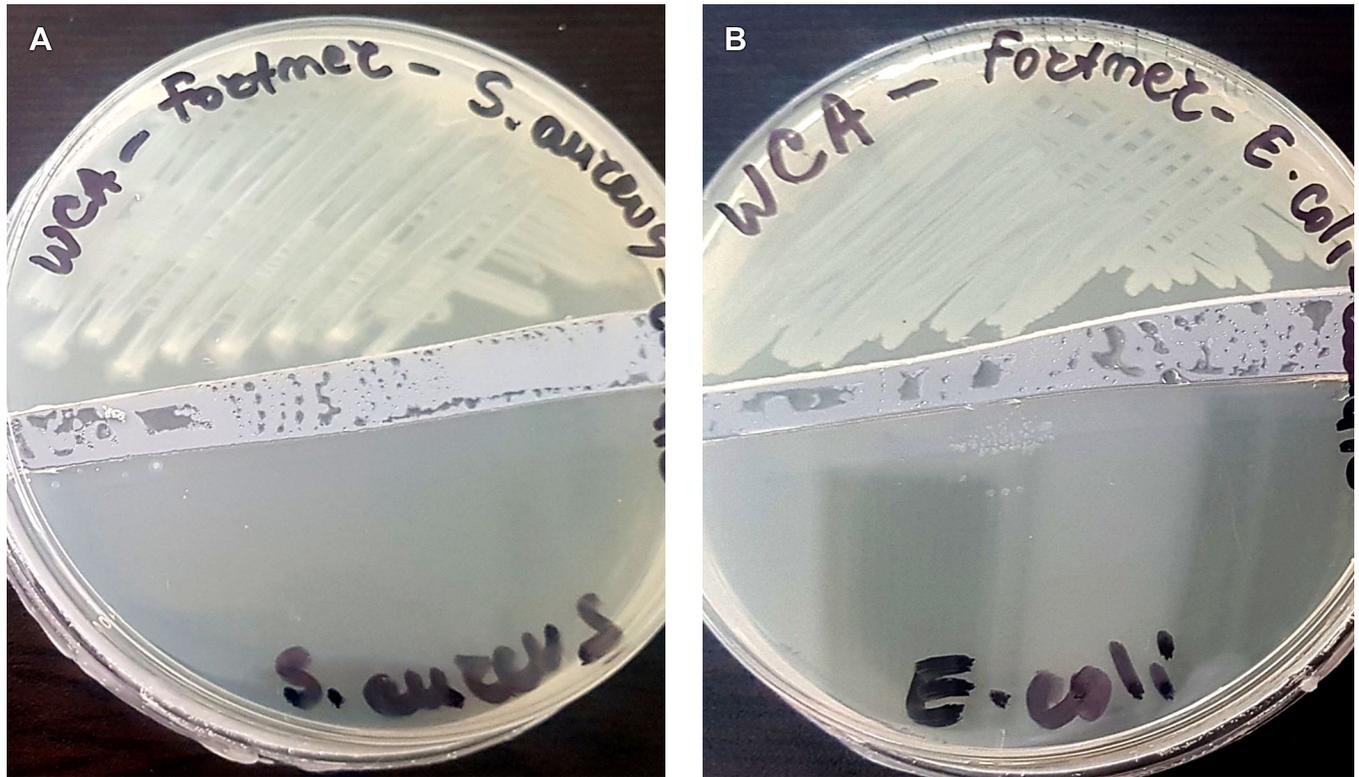
Each of the three methods used for anaerobic cultivation of *B. fragilis* proved to be successful. The degree of growth and colony size were similar in all three systems. Interestingly, in three of the six of the Wilkins-Chalgren agar plates, cultivated by the Fortner principle, the facultative anaerobe microorganisms *E. coli* and *S. aureus* were not successfully cultured. The reason is probably that this type of nutrient medium is intended for strict anaerobes. However, the target anaerobic microorganism had multiplied actively (Figure 1). This confirms the statement of Baughn and Malamy, 2004 [5] that genus *Bacteroides* are among the most aerotolerant anaerobes and, at certain oxygen concentrations, can survive and multiply. Sealing the plates with paraffin proved to be sufficient to create the necessary conditions for the survival of the strain.

It is necessary to specify that although *Bacteroides fragilis* ATCC 25285 showed heavy growths cultivated in a Candle jar system, this does not absolutely prove that this technique is suitable for all strict anaerobes. As discussed above, this method does not achieve complete anaerobiosis, and it is possible that the known aerotolerance of *Bacteroides* makes it suitable for this type of cultivation, unlike other anaerobes. Saha et al., 2016 [10] also share the opinion that the Candle jar is an alternative technique that can be used to reduce the major bulk of oxygen rapidly. However, they present a study in which they use a modified candle-jar system, acidified copper-coated steel wool is used for removal of residual oxygen after candle combustion.

CONCLUSION

Anaerobic cultivation of bacteria by chemical agents such as the Gas Pack system is a widely used method, and its high efficiency is well studied and known. However, achieving an anaerobic environment by Candle jar and Fortner principle are two alternative methods that are not being used or are being used less frequently or almost not at all. In fact, our study shows that these two methods effectively and with sufficient duration create conditions for the cultivation of strict anaerobe as *Bacteroides fragilis*. The Candle jar system and Fortner principle were found to be a sensitive and cost-effective alternative that might be used in resource-limited settings.

Fig. 1. *Bacteroides fragilis* ATCC 25285 (in the upper half of the nutrient medium) cultivate onto Wilkins-Chalgren anaerobic agar by Fortner principle. *Staphylococcus aureus* (A) and *Escherichia coli* (B) are seeded in the lower half of the culture medium. Although the oxygen-consuming microorganisms did not survive and accordingly did not consume the oxygen in the Petri dish, *Bacteroides fragilis* which is a strict anaerobe, demonstrate growth.



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