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Research Article

Analysis of Four-Hours Active Sampling Using 90 mm Plates + LTHT

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Abstract

Environmental monitoring is a key component to ensure the quality and safety of production processes in controlled environments, as required in pharmaceutical manufacturing areas. Various monitoring systems are available to verify compliance of controlled contamination environments, including active air sampling. This technique involves the use of plates containing Tryptic Soy Agar (TSA) for total bacterial count and devices that aspirate a known volume of air (1 m^3) through a perforated lid. This study focuses on continuous active air sampling using 90 mm plates and Active Air Sampler (AAS) devices, in accordance with ISO 14698:1-2003. The aim of this study was to increase the sampling time from 2 to 4 hours and the air volume from 1 m^3 to 2 m^3. At the end of the aspiration process plates was examined morphologically, to assess potential dehydration, and microbiologically for growth promotion capability. The results obtained after 4 hours of operation and 2 m^3 of sampled air showed no signs of dehydration, as no reduction in agar thickness was observed.

Furthermore, there was no impairment of the microbial growth or recovery properties. Therefore, the morphological integrity and nutritional characteristics of the plates remained unchanged, which was crucial for understating the impact of active sampling on the plates and for ensuring the reliability of microbiological data obtained during environmental monitoring.

In conclusion, the study met the acceptance criteria required for environmental monitoring, including: i) microbial growth compliant with incubation conditions on all plates; ii) recovery of microorganisms ranging between 50% and 200%; iii) absence of dehydration phenomena.

Keywords: Environmental Monitoring; Active Air Sampling; Dehydration Phenomena; Growth Promotion; Recovery

Introduction

Environmental monitoring

Environmental monitoring makes possible to assess the concentration of microorganisms' present, exclude the presence of potential pathogens and evaluate the effectiveness of the measures adopted to mitigate risk (Lgs 81/08) [1]. In a pharmaceutical company, environmental monitoring is considered a critical aspect as it is necessary to ensure the quality, safety and compliance of the products – requirements demanded by Regulatory Authorities (EU GMP Guide, USP <1116>, Food and Drug Administration, ISO 14698:1). This implies the measurement and control of specific

environmental parameters such as temperature, humidity and airflow within production areas, storage zones and laboratories, in order to: i) prevent contaminations; ii) to ensure regulatory compliance; iii) maintain sterile conditions; iv) support process validation. Preventing microbial contamination is one the primary goals of environmental monitoring, as identifying bacteria, fungi and other microorganisms in the environmental allows prevention of product contamination. Microorganisms can cause significant health risks and reduce product efficacy. Therefore, monitoring cleanrooms, collecting data and examining trends allows the microbiological state of the environment to be controlled [2]. Compliance with regulations is another essential aspect, as it is necessary to meet the requirements of Good Manufacturing Practices (GMP) and the standards imposed by regulatory agencies such as Food and Drug Administration (FDA) and European Agency of Medicine (EMA), which mandate continuous monitoring of production and laboratory environments. Sterility must also be guaranteed, essential for sterile drug manufacturing where even minimal microbial contamination can compromise product safety and efficacy. Thus, monitoring ensures conditions are appropriate for aseptic processes, preventing microbial contamination of final products. Finally, process validation support ensures that environmental conditions foster consistent and reproducible production processes [3]. However, monitoring and environmental control are two distinct concepts: i) environmental control involves the design of processes and parameters required to maintain the environment; ii) monitoring confirms environmental conditions and must be carried out during sterile operations. There are various monitoring methods and system used to verify and confirm the compliance of sterile environments [4], which is essential due to the many ways microorganisms can settle in critical areas including: i) air and surface. The ventilation system in production facilities is designed to minimize the survival, distribution, proliferation and growth of microorganisms. Hence, microbiological air sampling is viral to verify the effectiveness of cleaning and sanitation programs, personnel gowning procedures to detect and enumerate microorganisms and/or particles within cleanrooms. ii) personnel. Individuals working in aseptic environments are one the main sources of contamination, acting as carriers of microorganisms and generators of particles. iii) water. the most used solvent in pharmaceutical production and formulation. Due to its chemical properties, it can dissolve or suspend different compounds, including contaminants that could pose a health risk or react with active ingredients. iv) equipment and facility design. Materials used must be properly cleaned and sanitized, as they can offer favorable environments for microorganism survival and growth [5]. Given all the above, it is essential to emphasize the importance of executing environmental monitoring, especially during sterile operations. Monitoring in aseptic environments includes: i) air environmental monitoring; ii) surface environmental monitoring; iii) personnel environmental monitoring. Additionally, water used in parenteral preparations must be monitored, along with key microbiological parameters in critical areas, such as temperature, pressure and humidity [3,6].

Air sampling

When setting up monitoring processes, the timing and duration of sampling must be designed so as not to create unfavorable conditions during production phases that could affect product quality. Microbiological air sampling must be performed by authorized personnel in order to reduce the occurrence of false positive and minimize risk. There are two main air sampling methods: i) active or volumetric sampling; ii) passive sampling.

Active or volumetric sampling is the most widely used method and enables the detection of airborne microorganisms with an approximation of 70-80%. It uses devices such as Surface Air System (SAS), which channel a known volume of air (1 m^3) through a perforated lid positioned above a 90 mm Petri plate or a 55 mm contact plate containing a non-selective medium-Tryptic Soy Agar (TSA)- for total bacterial count. The sampling correlates the number of microorganisms to a known volume of air and results are expressed as CFU/m^3 [7,8].

Air sampling is performed by placing the SAS on a stable support at a specific point, at the height of 1 meter with the head oriented to simulate the position of an operator. After inserting the plate into the holder, securing the sampling head and selecting the desired air volume, the airflow is directed onto the agar surface and aspiration occurs at a constant rate for a defined time. Active sampling captures confined air and minimizes microorganism distribution differences caused by air currents, temperature and aerosol aggregate size. However, its duration must be kept short to avoid agar dehydration.

Passive sampling measures the deposition rate of airborne microorganisms carried by solid or liquid particles. It involves the use of settle plates containing nutrient agar, which are exposed in critical areas for a maximum of four hours, as established by Annex 1. Results are expressed as CFU/plate/time. It is defined as passive because it does not provide an absolute number of airborne microorganisms but only those that have sufficient weight and size to settle on the surface during the exposure period. When properly positioned, settle plates can estimate the quantity and frequency of microbial deposition on surfaces, including exposed products. The passive method has been standardized using the Index of Microbial Air contamination (IMA), which corresponds to the number of CFUs counted on a 90 mm Petri dish exposed using the 1/1/1 scheme: for one hour, one meter above the ground and about one meter away from obstacle and wall [6,9].

Surface sampling

Surface contamination is caused both by the deposition of airborne bioaerosols and by contact with personnel and contaminated materials. Sampling is carried out at the end of production processes in order to minimize the risk of contaminating materials and products during monitoring activities. Surface contamination is assessed using 55 mm contact plates containing raised nutrient media (TSA with or without neutralizing agents), which form a convex surface allowing microorganisms to adhere from smooth surfaces through a contact time of approximately ten seconds. Results are expressed in CFU/plate.

For sampling irregular surfaces or those in direct contact with the product (e.g. fill needles), swabbing techniques may be used. Swabs consist of a cotton or synthetic material affixed to a stick [6,7].

Personnel monitoring

Microorganisms are part of the normal human flora; therefore, personnel operating in aseptic environments are considered one of the main sources of contamination, as they act as carriers of microorganisms and generators of particles that may cause microbial contamination.

To minimize this issue, personnel authorized to operate in aseptic environments must strictly adhere to gowning and hygiene procedures approved by the Quality Assurance department. It is essential that personnel wear specific garments and protective equipment to access cleanrooms.

Sampling is performed at the end of sterile activities using 55 mm contact plates applied to five specific points: forehead, chest, right and left forearms and mask. 90 mm plates are used for glove sampling [3].

Continuous Air Sampling: ISO 14698-1:2003

Environmental monitoring refers to microbiological tests performed to detect the evolution of microbial counts and microbial flora growth within cleanrooms or controlled environments. It is important to remember that in Grade A areas (cleanrooms, isolators or RABS), maintaining microbial control through understanding contamination sources is essential [10].

As discussed previously, there are various environmental sampling methodologies, mainly based on two approaches: passive and active monitoring. The latter can be performed through either single-point air sampling or continuous air sampling. The difference is that single sampling provides a snapshot of contamination in a specific area, whereas continuous microbiological monitoring offers a deeper understanding of microbial presence and potential contamination risks during product manufacturing [11].

To choose the most appropriate sampling device, different Standards must be considered.

ISO 14698-1:2003 standards require that the sampler or Active Air Sampler (AAS) must efficiently collect airborne particles on a suitable culture medium, both biologically and physically [12].

Biological efficiency refers to the sampler's ability to collect dispersed particles while preserving the biological properties of the sample. This depends on the sampler's design, operating mechanism, duration of sampling and environmental parameters such as temperature.

Physical efficiency refers to the device's ability to collect particles of various sizes efficiently. Several factors influence physical efficiency, including the geometry of the head, its length and width, the diameter and number of slits, the airspeed and the precision of the slits between the sampler head and plate surface [12,13].

ISO 14698-1:2003 also requires that air sampler must be capable of: i) sampling a sufficient air volume within a reasonable time frame; ii) collecting 1 micron particles (with a d50 value of 1 micron); iii) maintaining unidirectional airflow within the room; iv) avoiding contamination of the surrounding area.

AAS devices are generally sieve-type samplers designed to achieve airflow speeds sufficient to obtain a d50 value of 1 micron, thus meeting one of the ISO 14698-1:2003 requirements.

The d50 value is defined as the mean particle size – meaning that if a sampler has a d50 of 1 micron, there is 50% chance that a 1-micron-sized organism will be impacted and collected [12].

There are various sieve sampler models, each with specific aspiration characteristics (ranging from 28.3 to 100 liters per minute). However, it is important to note that sieve samplers work in intervals – meaning the plate inside must be changed after each aspiration. This is due to the fact that repeated airflow impact on the same agar surface can cause dehydration reducing biological efficiency.

Sieve samplers designed for continuous sampling are built into a structure that places the sampling head at a fixed point, with the control unit and vacuum pump located outside the cleanroom (Figure 1).

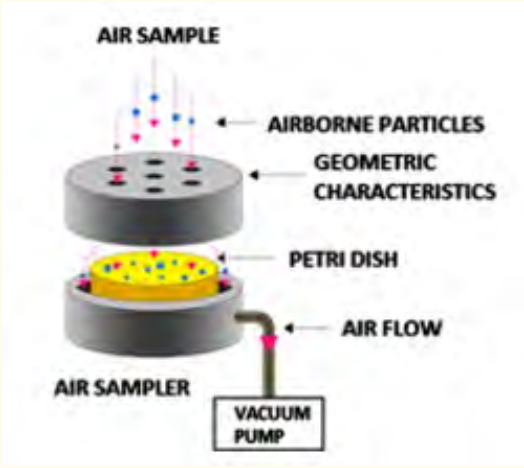

Figure 1: Continuous active air sampler.

Design requirements of air samplers in Grade A controlled environments include: i) d50 value of 1 micron; ii) high biological efficiency; iii) appropriate collection efficiency for the area's grade; iv) sufficient sampling duration; v) minimal human intervention; vi) broad variety of usable culture media.

A sampler's ability to recover possible contamination is defined by the Performance Rating (PR), calculated as the concentration of contamination the sampler can recover for a defined air concentration. The PR is computed using the following equation [14,15].

Performance Rating (PR) = n / (t*r*η)

n = minimum number of microorganisms

t = sampling time

r = air sampling rate

η = sampler collection efficiency

Annex 1: Cleanrooms and continuous air sampling

Cleanrooms are sterile environments designed to minimize the introduction and generation of particles within them, ensuring optimal conditions for pharmaceutical product manufacturing. Their design and management are regulated by international standards that define the procedures for initial qualification and continuous monitoring to maintain microbiological and particulate control [16]. Initial cleanroom qualification involves assessing air quality under static conditions to ensure that physical and microbiological parameters are adequate. Annex 1 of the EU GMP focuses on classifying production environments, as maintaining separation between production areas is crucial for contamination control. To ensure good air quality, a pressure differential of 12.5 Pascal is recommended. Pressure is a critical factor in cleanroom design – these environments are generally pressurized to protect exposed products from potential contamination sources. Positive pressure keeps particles out by pushing them toward lower-classified areas [8]. Temperature and humidity are also parameters that may vary depending on the processes and products involved. Therefore, they must be kept at appropriate levels, as humidity can be considered a risk factor for the product due to human perspiration and condensation [17]. Cleanroom classification is defined by the maximum allowable particle counts and microbiological contamination levels, expressed in colony-forming units (CFU). This is crucial for managing contamination risks that

could compromise product safety and quality. Therefore, a proper monitoring program for sterile areas is essential to ensure that production environments and cleanrooms maintain microbiological and particulate contamination levels within established limits. The classification of cleanrooms is described in detail in Chapter 1 of the EU GMP Annex, which states: "each manufacturing operation requires an appropriate level of environmental cleanliness during operation to minimize the risk of contamination – whether particulate or microbiological – of products or materials used". Annex 1 of the EU GMP and ISO 14644 provide guidance for cleanroom classification, specifying the particle size ranges to be monitored (from 0.5 to 5 microns), both at rest and in operation. The regulations also outline the minimum number of required sampling points and their distribution across the environment. Grade A and B areas require more frequent sampling than Grade C and D, which demand a broader sampling point distribution to cover the entire area. Microbial concentration in cleanroom is established during their initial qualification through a detailed risk assessment. The maximum allowable microbial contamination limits for each grade are defined during this process. Specifically, the EU GMP Annex 1 requires that Grade A areas must show zero detectable microbial contamination, meaning no CFUs should be present. The most recent revision of Annex 1 emphasizes the importance of continuous monitoring in Grade A aseptic environments (via air sampling or settle plates), stating that this must be performed "throughout the entire duration of critical operations, including aseptic set-up and processing" [6,18]. Continuous air sampling is an essential upgrade for industries with stringent environmental control requirements, where detection of production area contamination could pose a public health risk. Therefore, the importance of continuous sampling is often emphasized, as it is a slow form of sampling carried out at a specifics points that helps reduce the risk of contamination and is considered the most effective way to detect potential microbiological contaminants. The first step in approving continuous environmental monitoring is to audit each production area, analyzing factors such as i) equipment and processes at every production stage; ii) detection system effectiveness in alerting operators to potential contamination; iii) personnel training and behavior. Finally, reports on monitoring methods and frequency must be prepared, providing details on parameters such as pressure differential, temperature, humidity, airflow directionality and microbial surface contamination. Data obtained from environmental monitoring provide insight into the construction and ventilation of cleanrooms, the HVAC system, cleaning protocol and staff behavior [11].

Materials and Methods

Isolation of microorganisms

The microflora commonly isolated in pharmaceutical production environments primarily devices from human skin. These are mainly Gram-positive microorganisms, with a smaller proportion of Gram-negative ones. Although Gram-negative organisms are less frequently isolated, it is nonetheless necessary to include at least one strain in microbiological studies. For this study, seven microorganisms were selected: five standard reference strains provided by the American Type Culture (ATCC) and two "wild-type" strains recovered from environmental monitoring activities (Table 1).

Culture medium	Test microorganism	Temperature	Time	Acceptance criteria
Tryptic Soy Agar w. LTHT	*S. aureus* ATCC 6538	32,5 +/- 2,5 °C	≤ 3 days	-50%; +200%
	P. aeruginosa ATCC 9027	32,5 +/- 2,5 °C	≤ 3 days	-50%; +200%
	B. subtilis ATCC 6633	32,5 +/- 2,5 °C	≤3 days	-50%; +200%
	C. albicans ATCC 10631 *A. Brasiliensis* ATCC 16404	20-25 +/- 2,5 °C	≤5 days	-50%; +200%
	**S. epidermidis* **Micrococcus luteus*	32,5 +/- 2,5 °C	≤3 days	-50%; +200%

Table 1: Microorganisms and fertility testing for general-purpose solid media. Wild-type microorganisms isolated from environmental monitoring.

Active continuous air sampling using 90 mm TSA plates with LTHT

Three batches of 90 mm TSA plates containing 30 mL of nutrient medium were selected (Table 2). Each plate was placed inside operation cones located at specific continuous monitoring stations within the cleanroom, for a maximum sampling duration of four hours. The aspiration process was manually activated through dedicated software located outside the cleanroom, while maintaining environmental conditions typical of a production process. At the end of each sampling cycle, the plates were removed from the cones and transported to the Microbiology Laboratory for: i) morphological evaluation of assess any dehydration phenomena; ii) growth promotion testing (GPT) to verify nutrient properties.

For morphological evaluation, comparisons were made between: i) aspirated and non-aspirated plates with the same nutrient volume (30 mL); ii) plates containing approximately 15-20 mL of nutrient medium, typically subjected to a maximum aspiration time of two hours.

Growth promotion test

Prior to use, each purchased batch of plates underwent sterility verification. Five plates per batch were incubated for three days at 30-35 °C. Following sterility testing, the study evaluating the growth promotion properties and recovery capabilities of the inoculated microorganisms was initiated. Microbial suspensions were prepared according to European Pharmacopoeia guidelines, with a maximum of five passages from original cultures. Serial dilutions were performed in peptone buffer pH 7 (VWR Chemicals) to achieve the desired concentration.

Plates	Batch	Composition	Quantity/liter
TSA 90 mm	201600	Peptone from Casein	15 g/l
w. LTHT	141478	Soya Peptone	5 g/l
	141468	Sodium Chloride	5 g/l
		Agar	15 g/l
		Polisorbate	5 ml/l
		Lecithin	0.7 g/l
		Istidine	0.5 g/l
		Sodium thiosolfate	0.3 g/l

Table 2: Composition of culture medium.

From each microbial suspension, 0.1 mL was inoculated onto the plates, distributing less than 100 CFUs uniformly over the agar surface using an L-shaped spreader. After incubation (three days for bacteria, five days for fungi) at 30-35 °C and 20-25 °C, respectively, results were read. Fertility was considered compliant if the mean colony count per microorganism did not differ by more than a factor of two (-50%; +200%) compared to positive control plates.

Results

This study highlighted two key aspects: i) the fertility of the media and the recovery of microorganisms; ii) the dehydration of media after aspiration.

All media inoculated with the standard microorganisms showed microbial growth within the expected incubation times and temperatures. Therefore, the recovery rates for each microorganism across the three TSA batches complied with the requirements of the European Pharmacopoeia, showing a recovery of at least 70% of the inoculated sample (Table 3; Figure 2).

	Batch	*B. subtilis* ATCC 6633	*S. aureus* ATCC 6538	*P. paraeruginosa* ATCC 9027	*Micrococcus luteus*	*S. epidermidis*	*C. Albicans* ATCC 10231	*A. brasiliensis* ATCC 16404
TSA 90 mm w. LTHT	201600	1)58	1)117	1)52	1)103	1)126	1)126	1)62
	141478	2)91	2)83	2)64	2)114	2)121	2)107	2)53
	141468	3)84	3)96	3)55	3)78	3)130	3)118	3)60
K	201600	1)66	1)108	1)63	1)110	1)122	1)131	1)58
	141478	2)93	2)71	2)75	2)109	2)123	2)115	2)49
	141468	3)75	3)94	3)67	3)96	3)133	3)127	3)51
% recovery	201600	1)87,9%	1)108,3%	1)82,5%	1)93,6%	1)103,3%	1)96,2%	1)106,9%
	141478	2)97,8%	2)116,9%	2)85,3%	2)104,6%	2)98,4%	2)87%	2)108,2%
	141468	3)112%	3)102,1%	3)82,1%	3)81,3%	3)97,7%	3)92,1%	3)117,6%

Table 3

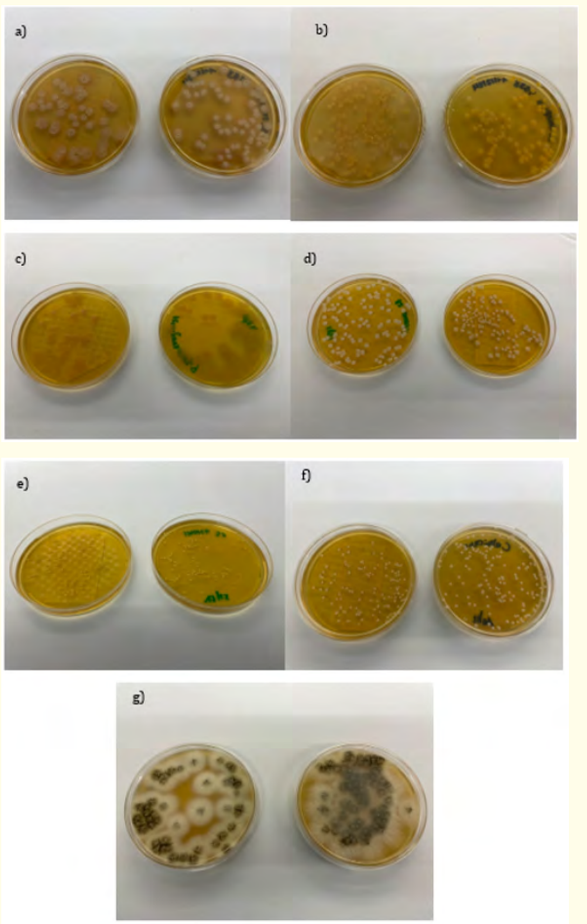

Figure 2: GPT and recovery of microorganisms; a) *B. subtilis*; b) *S. aureus*; c) *P. aeruginosa*; d) *Micrococcus luteus*; e) *S. epidermidis*; f) *C. albicans*; g) *A. brasiliensis*.

Another key aspect evaluated was the dehydration of the medium. TSA 90 mm plates with neutralizers, containing 30 mL of medium, and inserted into aspiration cones for up to four hours, were compared with: i) non-aspirated plates containing the same amount of medium; ii) plates containing 15-20 mL of medium, generally subjected to a maximum aspiration time of two hours.

The thickness of the medium was measured on plates subjected to different aspiration times. The results showed that plates with 30 mL of medium, despite longer aspiration times, did not exhibit significant thickness reduction (Figure 3), unlike plates with 15-20 mL of medium, exposed to airflow for two hours, which showed a significant reduction in thickness due to dehydration phenomena (Figure 4 a-b).

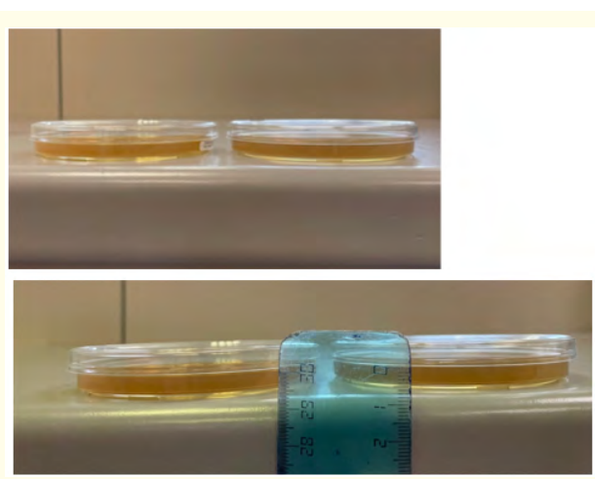

Figure 3: Comparison between 90 mm plates containing 30 mL of culture medium: with and without air aspiration.

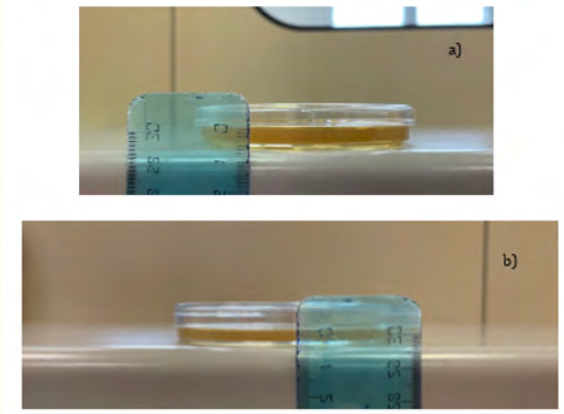

Figure 4: Comparison between 90 mm plates containing: a) 30 mL of nutrient agar; b) 15-20 mL of nutrient agar, exposed to 4 hours of active air sampling.

Discussion and Conclusion

Continuous air monitoring within aseptic environments is fundamental to ensuring controlled and safe conditions, especially where airborne contamination could negatively impact product quality. In cleanrooms, the concentration of suspended particles, as well as temperature and humidity, must be maintained within extremely strict limits.

Continuous air monitoring uses advanced systems that allow constant surveillance of the environment during the entire production phase, detecting and quantifying airborne microorganisms such as bacterial and fungal spores. It differs from traditional monitoring methods by providing a continuous measurement, thus improving the forecasting of air quality and allowing early detection of any increase in microbiological contamination. This enables prompt corrective actions to maintain environmental compliance with quality standards such as those established by ISO 14698 [19,20].

However, it is important to consider that a continuous airflow over the culture medium may reduce the humidity of the medium by accelerating water evaporation, leading to dehydration of the agar. Dehydration of microbiological culture media is a critical issue as it affects the growth, survival and activity of microorganisms. Culture media are characterized by specific concentrations of organic and inorganic substances that must be hydrated to provide a suitable environment for microbial proliferation.

Dehydration can disrupt this ecosystem, impairing microbial growth and modifying microbial interactions. Furthermore, it can lead to detrimental effects, such as reduced enzymatic activity essential for nutrient metabolism or induce stress conditions that cause cell death or inhibit microbial growth [21,22]. Breeuwer in "Adaption of pathogenic microorganism to dry conditions", demonstrated that some microorganisms can respond to dehydration by activating specific adaptation mechanisms, such as synthesizing osmoregulatory compounds that protect cells from dehydration stress [23].

However, the capacity to adapt varies across species and depends on environmental conditions, such as airflow speed and dehydration exposure time. As previously mentioned, traditional air monitoring system involve aspirating 1 m^3 of air using devices equipped with perforated lids. In this study, the goal was to investigate possible morphological changes after a maximum aspiration period of four hours.

The results showed that aspirating air using 90 mm TSA plates with LTHT containing 30 mL of medium, even for extended periods up to four hours and volumes up to 2m^3, did not cause dehydration phenomena. Regarding the growth promotion properties and microorganism recovery, the results confirmed that the use of non-selective media maintained these properties even under prolonged exposure and higher air volumes compared to traditional conditions. Thus, morphological evaluation and nutritional characteristics were crucial to understanding the impact of active sampling on the plates and ensuring the reliability of microbiological data obtained during environmental monitoring.

In conclusion, this study met the acceptance criteria required for environmental monitoring, including: i) microbial growth compliant with incubation conditions on all plates; ii) recovery rates between 50% and 200%; iii) absence of dehydration phenomena, confirming the suitability of these plates for continuous active monitoring for up to four hours.

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