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Challenges of growth-based microbiological methods in sterility assurance of pharmaceutical product manufacturing

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Abstract

Nonsterile parenteral products cause harm to patients, resulting in serious public health issues and great financial damage to drug manufacturers. To avoid such consequences, a contamination program should be deployed to ensure product sterility, and this can include periodic bioburden quantification and sterility testing. The use of traditional growth-based microbiological methods alone described in the current compendial guidelines for bioburden and sterility testing have been argued as unsatisfactory to determine the absolute absence of microorganism. This review aimed to investigate the advantages and drawbacks of the current conventional and the rapid microbiological detection methods. An inclusive review of literature was conducted to describe the microbial contamination in the sterile manufacturing of pharmaceuticals. An overview on the challenges and limitations of the traditional growth-based methods for bioburden assessment and sterility testing was presented. The conventional growth-based bioburden and sterility testing methods are the current mainstay technology in sterile pharmaceutical manufacturing. However, the rapid microbiological detection methods provide highly sensitive systems capable of verifying the absence of microbial contaminants including those with negligible counts of microbes in a short period of time, which might outweigh those of growth-based methods. Adoption of the rapid microbiological detection methods should be reconsidered as a future challenge in improvement of the sterility assurance of pharmaceutical products. However, their application in the large-scale manufacturing of pharmaceutical products may remain lagging because of limitations such as high expenses, the expertise, cost of test validation and the regulatory restrictions.

Keywords Bioburden, Sterility assurance, Sterility testing, Growth-based methods, Rapid microbiological methods, RMM

1 Introduction

The presence of bioburden and sterility assurance are crucial issues in the sterile manufacturing of pharmaceuticals. Failure to control unexpected bioburden levels in pharmaceutical production can create serious health risks [1, 2]. These risks ranging from

severe complications to life-threatening conditions. For instance, contaminated ophthalmic solutions causes endophthalmitis and risks for vision loss, non-sterile parenteral can introduce systemic bloodstream infections which results in bacteremia or septicemia, or septic shock if endotoxin contamination is involved.

Strict adherence to the regulatory requirements of aseptic processing techniques, good manufacturing practices (GMP), and sterilization are fundamental to ensure the sterility of such products. However, with standards and guiding regulations in place [3], microbial contamination remains an existing problem in the sterile manufacturing of pharmaceutical products [4, 5]. Secondary to health consequences, failure to ensure sterility leads to the recall of released products [6–9] which is cost burdens to manufacturers [10] and healthcare providers [11]. Data from published reports revealed that most of the recalled drugs were due to a lack of sterility [8, 12].

Most of the reported incidents of microbial contamination in sterile manufacturing refer to raw materials, including pharmaceutical water [13, 14], the production environment, and the manufacturing process impacted largely by human intervention as the major sources. High bioburden levels are expected to be unpredictable when aseptic manufacturing, GMP, and bioburden control are applied. Nonetheless, the bioburden on the finished sterile product should be eliminated by sterilization or kept at the allowable limits using aseptic processing.

Estimation of bioburden intensity and identification of contaminant microorganisms are often mandatory to avoid harm to consumers. In products intended to be sterile, such as parenteral and ophthalmic preparations, the presence of unexpected high levels of pathogenic or non-pathogenic organisms in the manufacturing process is impermissible and requires immediate remedy. Elimination of bioburden acquired in the manufacturing process is controlled by aseptic processing, sterile filtration or terminal sterilization; hence the end-product must be sterile.

In non-sterile pharmaceuticals taken orally or topically, such as solid dosages (e.g., tablets and capsules), the bioburden limit varies according to the mode of application, but must not exceed the limits specified by regulations, e.g., United States Pharmacopeia (USP) [15, 16]. Therefore, prevention or mitigation of bioburden contamination is vital and can be achieved by bioburden control and sterility assurance to ensure a high microbiological quality of products.

The challenges in bioburden control and sterility assurance are often estimated using growth detection techniques used in bioburden estimation and sterility testing [17]. Traditionally, bioburden estimation and sterility testing in the pharmaceutical industry have been performed using compendial microbiological methods, relying heavily on manufacturing environmental samples and end-product testing to determine the existence of microorganisms in test samples after culturing in growth medium [18]. These growth-based detection techniques are dominant with compendia and are easy to apply with reasonable costs, but have several limitations. The most arguable limitations include the following: inefficiency in detecting all microbial contamination, inability to discriminate between viable and non-viable microorganisms [19, 20], and time-consuming [21]. A significant challenge is the consequences of growth-based techniques, namely, false-positive and false-negative results [22].

This review provides background information on the microbial contamination of pharmaceuticals and presents the conventional growth-based bioburden and sterility

test methods. The review also discusses the limitations of growth-based microbial methods for bioburden and sterility test in pharmaceuticals manufacturing and contrast it to the advantages of rapid microbial detection methods in terms of accuracy, specificity, and time to results attributes.

2 Microbial contamination of the manufactured pharmaceutical drugs

2.1 Pharmaceuticals

Pharmaceuticals are manufactured in compliance with GMP and pharmacopeial guidelines to ensure standard microbiological quality. Sterile drugs such as injectables in contact with the bloodstream and ophthalmic drugs applied directly onto the eye surface are expected to have no bioburden [23]. Non-sterile pharmaceuticals applied to non-sterile regions with local microbiota, such as the mouth, nose, and skin, have variable permissible limits of bioburden specified by regulations, for example, ≤ 1000 colony forming units (CFUs)/gram or milliliter in raw materials [15], provided that they are free of objectionable microorganisms such as *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E. coli*), and *Candida albicans* [16]. Many pharmaceutical products are acceptable with low bioburden or preserved by addition of chemical compounds to prevent microbial proliferation without compromising the product's quality. Microbial growth of non-sterile products can be controlled by maintenance of temperature, humidity and other factors during storage [24]. The delayed detection of the in-process or post-release bioburden leads to the proliferation of microorganisms and exacerbation of the microbial contamination problems [25, 26].

The detection of substandard microbiological quality medicine occurs in many parts of the world [11, 26]. This is evidenced by the number of drug recalls reported in the literature equally in developed and developing countries [8, 11, 12, 27, 28]. A study analyzing US Food and Drug Agency (FDA) recalls between 2017 and 2019 revealed that 83.7% of drugs were recalled because of a lack of sterility [29].

3 Bacterial contamination of drugs

Pharmaceutical products can be contaminated by both Gram-positive and Gram-negative bacteria [30]. Bacteria can survive on wet and dry surfaces, dust, dirt, and in a humid environment. Gram-positive bacteria, aerobic and anaerobic spore formers, can survive in dry and stressed environments such as in cleanrooms [31], while, Gram-negative bacilli prefer a moisture-associated environment and surfaces; nonetheless, some Gram-negative bacteria can survive desiccated conditions for weeks and a source of high risk of endotoxin production when the growth conditions are appropriate. In general, the survival duration of both bacterial classes can extend from weeks to months [32–34]. Elimination of such contamination including objectionable microorganisms is crucial in manufacturing of sterile products, whilst, complete sterility is not required for non-sterile products, but testing for objectionable microbes including *Burkholderia cepacia complex* (BCC) is critical to avoid serious infection particularly in vulnerable patients, as well as to avoid drug recall.

The FDA reported that recalls of non-sterile drugs released into the US market in the period 2012–2019 were due to different bacterial contaminants, mostly Gram-negative bacteria. BCC was the predominant identified species (102 recalls), followed by *Ralstonia pickettii* (45 recalls) and *Salmonella species* (28 recalls). The *Burkholderia* bacterial

genus is frequently identified in most recalls as a water contaminant of pharmaceutical formulations [2, 9]. Of the reported recalls, 52 incidents were attributed to the yeast and mold contamination in non-sterile drugs [8].

4 Fungal contamination of drugs

Fugal contamination of drugs is less common than bacterial contamination. According to FDA data, fungal contamination of pharmaceuticals is involved in approximately 20% of all recalls [5, 35]. In 2014–2015, FDA investigations revealed only 15 fungi-related recalls out of approximately 2500 [5]. Between 2004 and 2011, only 23 recalls related to yeast and mold contamination were reported out of 142 recalled microbially contaminated products. In 2012–2013, a serious large outbreak in the US was associated with the extremely antifungal resistant form of "black mold" *Exserohilum rostratum*, a variety of melanized fungi [36]. This fungal outbreak resulted in 753 patients suffering serious complications of fungal meningitis and spinal/paraspinal infections, of which 8% of patients died [1, 37]. Fungal contamination of the manufacturing environment, especially in the clean room, poses a great risk of batch contamination and rejection, as well as a threat to drug consumers once undetected by air and surface monitoring regimes [36].

5 The current microbial contamination testing methods

5.1 Bioburden monitoring in sterile manufacturing

According to the Pharmacopeial definition, bioburden refers to the total aerobic microbial count (TAMC) and total combined yeast and mold count (TYMC) on product. The bioburden count is measured in CFUs and the bioburden test is often performed for every batch of both sterile and non-sterile products. Bioburden count for products intended to be sterile such as aseptically filled/filtered pharmaceuticals and single use products is mandatory as a control prior to final filtration or sterilization [16] .

In sterile production, bioburden testing provides a significant data for judging the product microbiological quality. Therefore, it is utilized at different phases of the manufacturing process to evaluate a new product or incoming raw material's microbial load, and in environmental monitoring programs. In routine environmental monitoring, bioburden testing is performed on a schedulable plan on predetermined areas, while non-routine monitoring is used in the events of bioburden deviation to confirm results. Data from environmental monitoring is helpful in applying corrections and prevent occurrence of product contamination [38].

In non-sterile manufacturing, the bioburden information includes viable aerobic microbial count and type, and is useful in leading the manufacturer in case of specific microbial load was out of limit, or presence of objectionable bacterial growth. Bioburden monitoring can be indicative of the root cause of contamination. Sampling for bioburden testing is performed at various stages from raw material to finished product routinely, for every production batch, especially for products manufactured with aseptic processing and not terminally sterilized.

5.2 Sampling for bioburden testing

Bioburden samples are taken from the anticipated sources of contamination such as raw materials, production environment, manufacturing process, people and water. For

bioburden control and sterility test, regulatory bodies such as FDA and European Medicines Agency (EMA) provides specific guidance on sampling of product batches. For example, pharmaceutical samples must be taken aseptically in a sterile container at the end of the process. The sampling plans involve recommended sample size and sampling methods [38]. USP < 1116 > details the important sampling sites, which mostly adjacent to the potentially expected high bioburden areas such as those in proximity to exposed product, closures and containers, hard to clean places, or from traffic areas responsible for microbial dispersal [39].

To further assess the impact of microbial contamination from the manufacturing environment on the product; the environmental monitoring programs uses some other microbiological tests to monitor the bioburden of air, surfaces, operators and equipment. These tests include settle plates, air sample, finger plate, swabs and contact plates [40]. Table 1, lists the basic bioburden environmental monitoring methods of the manufacturing process and their advantages and limitations.

5.3 Pharmacopeial bioburden testing

The current pharmacopeial bioburden estimation techniques involve membrane filtration, pour plate and spread plate methods. These bioburden techniques apply a number of culture media which have been described to be competent in microbial extraction and bioburden testing. The most commonly used media include TSB for the detection of aerobes, facultative anaerobes and fungi, and fluid thioglycolate medium (FTM) for the detection of aerobic and anaerobic bacteria [55]. In addition, solid agar media including TSA and plate count agar (PCA) are typically used for facultative aerobic bacteria. For cultivation of yeast and molds, media such as Sabouraud medium (liquid), Sabouraud dextrose agar (SDA), TSA, potato dextrose agar (PDA), are generally used [56]. Liquid (broth bottles) and solid (agar plates) media are used depending on the bioburden estimation standard methods.

5.4 Bioburden methods

For sterile products, during their manufacturing process, an understanding of bioburden is best practice. TAMC and TYMC are used to assess the CFU count of bacteria and fungi in liquid product samples. Non liquid samples are extracted using a validated method (e.g., rinsed in a 100mL of physiological saline), which is then aseptically filtered through 0.45 μm membrane filters. The filter retaining the microorganisms is placed on TSA agar and incubated at 30–35 °C for 3–5 days. For unfilterable samples, a certain volume (e.g., 1 mL) of the rinse can be transferred to TSA agar plates using either pour plate (liquid sample mixed with melted agar) or spread plate (liquid sample spread over solid agar surface) methods. Incubation temperature conditions are based on the manufacturing regimen. Lower 20–25 °C range is commonly used to encourage fungi growth, while the mid-range of 30–35 °C is for human skin microbiota (e.g. Micrococci and Staphylococci) and environmental contamination such as. *Bacillus spp*., and the 37 ± 1 °C is for recovery of human commensals and water contaminants (e.g. *Pseudomonas*, *Burkholderia*, etc.), the latter temperature is not commonly used because it may give lower mean counts comparing to the former two ranges [38]. After the incubation period, the average CFU/mL or 100 mL per product item is obtained from plates by visual counting [57]. The product is considered compliant to defined specifications

Table 1 Advantages and limitations of bioburden environmental monitoring

Microbiological method	Test target	Method description	Advantages	Limitations	References
Settle plates (also defined as fallout plate) and Passive air sampling.	Manufacturing air environment	Open tryptic soya agar (TSA) plates placed in different spots for a specific time duration maximally 4 h, where airborne microbes deposited on agar surface. Incubation: 35 °C/ 24–48 h for bacteria, and 25–30 °C/5–7 days for fungi. Result: CFU/4hours/90 mm plate.	Direct semi-quantitative and cost-effective testing of air microbial contaminations. Easy observation of results. Microbial colonies are available for further identification.	Agar loses moisture with prolonged exposure to air and dehydrated, thus, loses capacity to enhance growth. Long exposure/incubation time. Not quantitatively accurate. Can be easily affected by surrounding temperature, air current and humidity conditions. Not suitable for small size pollutants.	[40–43]
Active viable air sampling	Manufacturing air environment	Different methods exist, examples include: Centrifugal, uses agar strips. Filtration, air particles settle on a gelatin filter membrane, the membrane is aseptically placed onto a TSA plate and incubated. Sieve Sampler. Result: CFU/m^3	Different gamma sterilized, triple packed media and media volumes can be used.	Test disrupted by the airflow. Shear forces. Some systems are not suitable for Grade A area. Impaction ratio can be affected by air velocity in the gap between the sampling slit- to-agar surface. Risk of contamination during plate changing by operator.	[40, 42, 44]
Finger (touch) plates or glove print.	Personnel	Operator fingers imprints of both gloved hands onto the surface of the 90 mm diameter TSA plate, at the end of the preparation and before glove disinfection. Contact time (3 s). Result: CFU/ 5 fingers on both hands / gloves.	Provides informative information	Impractical with inappropriate agar strength.	[40, 45, 46]

Table 1 (continued)

Microbiological method	Test target	Method description	Advantages	Limitations	References
Contact plates	Flat dry surfaces of building, walls, equipment, containers and personnel's hood, arms and torso.	General or selective agar plates placed on surfaces to maximally collect microorganisms deposited from the environment or workers contact. For low bioburden. May contain neutralizing agent. Contact time (5–10 s) Result: CFU/4hours/55 mm plate.	Provide semiquantitative data. Easy to use. Can be made with selective agar for targeting certain microbes. Efficient than swabs for microbial recovery from cotton fibric. Efficient than dip slides and swabs for recovering of methicillin-resistant *S. aureus* (MRSA) from stainless steel surfaces.	Can be affected by the surface type, by which one application is insufficient to recover the microorganisms present. Can be affected by surface disinfectants. Some brand products have low microorganism recovery rate (range, between 23% and 56%).	[45, 47–49]
Swabs	Non flat equipment and surface and difficult to reach narrow locations	Swab samples taken from the test surface then immersed in tryptic soya broth (TSB) liquid medium tube and incubated. Suitable for high bioburden.	Simple, easy to use and affordable method. Most used sampling method. Made of different absorbent materials including cotton, polyester, calcium alginate, rayon, macrofoam, etc. Macrofoam and cotton swabs are more effective than other materials. Cotton swabs recovers more CFU especially form wet surfaces. Cotton swabs have high uptake efficiency of eluting buffers (95%).	One application per sample site may be insufficient. Sample extraction from the sampling device is required. Provide only qualitative results (no count). Result taken by visual observation. Less effective than RODAC plates for the detection of Gram-positive cocci. Cotton swabs have low cell release efficiency (< 50%), while, foam swabs have low uptake efficiency (57.9%). Results are highly variable because of difficulties to standardize swab sampling technique and swab material variability.	[45, 50–53]

Table 1 (continued)

Microbiological method	Test target	Method description	Advantages	Limitations	References
Dip slides	Solutions and flat & uneven surfaces.	Dip slides are dual-sides plastic paddles, coated on one side with TSA medium, and other media such as a MacConkey or a violet red blood glucose agar on the other side. Agar may contain neutralizers. No consensus on the contact time and pressing. Dipping in liquid is ~ 30 s. Pressing on surface, from ~ 5–15 s to 30 min. For low bioburden. Incubation: 35 °C for 48 h. Result: CFU/mL or CFU/CM^2.	Unlike contact plates, the slide square agar design ease sampling from confined spaces. Protected from external contamination by a plastic tube which maintain slide humidity, so prolongs agar slide shelf-life.	Results of CFU are determined basing on visual comparing to the supplier culture chart.	[45, 49]
Petrifilm	For wet, dry and uneven surfaces and also for finger dabs.	For low bioburden. Can be used to contact surfaces directly or loaded with a swab sample. Petrifilm paper is impregnated with media.	Quick and easy to use. Flexible to apply on non-flat surfaces. Can be an alternative to agar media for water samples and surfaces. Small volume of sample (1 mL). Needs less incubator space, less time, less materials and waste. Low cost and suitable for high volume of samples. Efficient than dip slides for recovering of *S. aureus* and MRSA.	Colony counting by naked eye is difficult for high bioburden samples. Colonies has no color and their morphology is not clear. Known contaminant is difficult to distinguish when appears in sample and control.	[45, 54]

based on the risk associated with the product (e.g., < 10 CFU/100 mL); in any result in high risk manufacturing processes, recovered CFUs should be identified to the species level to understand potential sources and risk mitigation [58]. Bioburden test methods should be validated for microbial limit testing before use on the actual samples to ensure effective recovery of microorganisms. To meet the GMP regulatory requirement, test validation has to be conducted in presence (suitability test) and absence (growth promotion test) of the product to be tested. The test is considered suitable for specified positive control samples when the CFU recovery is ≥ 50% and ≤ 200% compared to the positive control plates. Product samples containing preservatives or antimicrobial agents that are inhibitive to microbial growth can affect the reliability of bioburden test results. Therefore, samples must be sufficiently neutralized and maximum matrix interference

is allowed [59]. This is to adjust bacteriostatic/fungistatic abilities and to avoid false-negative results [22]. Neutralization of samples containing antimicrobial agent or probable disinfectant residues can be performed by sample dilution, filtration or enzymatic inactivation, dependent on the targeted preservative in the product. Polysorbate (tween 80) and soy lecithin are examples of neutralizing agents. Neutralizing agents must be validated for their effectiveness as neutralizers and any negative impact on growth themselves according to the USP < 1227>[60]. Additional challenging factors that can have an effect on the microbial contamination testing of manufactured product items include the operator, the sampling, the testing environment, the sample nature and size, the culture media, the microbial recovery technique and the incubation temperature and time duration.

5.5 Pharmacopeial sterility testing

Sterility in the pharmaceutical production is defined as the complete absence of viable (multiplying) microorganisms in the processed items [61]. Assurance of sterility is commonly confirmed by sterility testing of product samples using the most commonly used compendial tests detailed in the USP chapter <71>[23].

The traditional sterility test is performed by two different methods, the direct and indirect inoculation methods. In the direct method, the batch representative samples are aseptically transferred to a liquid culture of TSB (soybean-casein digest medium) and FTM followed by incubation. Whereas, in the indirect method, the sample is not in contact with the culture media, it is rinsed or solubilized in a recovery media (e.g., physiological saline), the suspension then filtered through not greater than a 0.22 µm sterile filter and placed on TSA plates and incubated [23]. The test results can be attained after 14-day incubation period at a designated temperature e.g. 35 °C for FTM and 25 °C for TSB, and is based on the visual inspection of the liquid medium turbidity or CFU growth on filters. The visual inspection during the incubation period can be scheduled e.g. for days 3, 5, 7 and 14 [62].

Sterility decision of a product batch is one of the most challenging aspects of the manufacturing process. Therefore, performance of sterility test is usually assigned to skillful operators and conducted in a sterile environment e.g. safety cabinet or an isolator to avoid false results. However, operators are responsible for 80–90% of the microbial isolates from cleanrooms [63]. The false-positive rate of sterility tests has been reported as high as 0.5%, and reasoned to be due to human error, manipulation, test environment, materials and incubation conditions [64].

6 Challenges of growth-based bioburden and sterility test

6.1 Sampling and microbial contamination distribution

Performing bioburden and sterility testing as the only measures to confirm absence of contaminant microbes is unsatisfactory in perceiving all contamination incidents for different reasons. Firstly, the standards' sampling procedures for bioburden monitoring or sample size are not adequate to provide sufficient information in monitoring of all potential risk points relevant to the product's microbiological quality [65], because the possibility of bioburden detection is dependent on sample size and the real proportion of contaminated items in the batch [66]. For example, the sample size as determined by the pharmacopeia for a batch of over 500 filled container items is only 20 samples,

which is far beyond to provide statistical confidence in sterility assurance of the produced batch [67].

Secondly, non-uniformity of microbial contamination distribution leads to false-negative test results which can be presumed due to the testing were performed on non-contaminated samples, or the test conditions are prohibitive to microbial growth. Here, the contaminated samples of the same batch are realized after distribution, when the microorganisms adapt to the product formulation and proliferate during storage [9]. Therefore, random sampling alone is not satisfactory for accurate detection of contamination in a manufactured batch [67]. Factors such as stress conditions of the aseptic environment and disinfectant residues on processing surfaces has also a significant influence on the microorganisms' type and distribution, and triggers some saprophytic and pathogenic bacteria to convert to non-cultivable dormant state [63, 66].

6.2 Viable but not culturable bacteria (VBNC)

VBNC bacteria can be present in adverse conditions of nutrition and temperature or antimicrobial/chemical preservative influence [20, 68]. In such a state, these bacteria can become small and undergo metabolic changes, leading to inability to form colonies on a culture medium and are undetectable under normal growth conditions. Microorganisms exposed to sublethal disinfection and sterilization agents can be damaged but still viable overtime. These injured vegetative bacteria or spores can require an extended lag phase to repair the damage and revive [69]. However, characteristics such as cell integrity, virulence, adherence to host cells and endotoxin production remain unchanged, and present a health risk when resuscitated [20, 70, 71]. Unfortunately, many VBNC bacteria are endotoxin producing Gram-negative pathogenic species and include *B. cepacia*, *Helicobacter pylori*, *E. coli*, *Listeria monocytogenes*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Salmonella typhi*, *Enterobacter cloacae*, *Aeromonas hydrophila*, *Campylobacter jejuni*, *Legionella pneumophila*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Vibrio alginolyticus*, *Vibrio cholerae*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. Gram-positive bacteria, such as *Enterococcus faecalis* and *Mycobacterium tuberculosis*, can also convert to VBNC [71, 72]. Gram-negative VBNC bacteria constitutes a great risk in sterile manufacturing due to firstly undetectability by growth-based methods [73] and secondly the possibility of endotoxin release when favorable conditions are available. Endotoxin contamination is life-threatening, therefore, endotoxin assays such as Limulus Amebocyte Lysate (LAL) test is an essential requirement in monitoring endotoxin levels during the manufacturing process for some products. Also, it is a release requirement for parenteral products (e.g. injections) to confirm the final product meets the standards of endotoxin limits.

The ability to recover of injured VBNC and dormant sporulated bacteria constitutes a great risk to the sterile manufactured products [69]. In many cases, these bacteria revive and proliferate under the right conditions while the products are in storage; therefore, contamination control strategies and verification test methods (including bioburden) should be considered for VBNC detection.

6.3 Culture media limitations

Culturing media plays a critical role in microbial detection and identification, and their use faces great challenges including for VBNC detection. Although, TSA, TSB, FTM,

SDA and other media were recognized as the recommended standard media for bioburden estimation and sterility testing [56, 74]. However, a report by the USP Modern Microbiological Methods Expert Panel-2017 [75] describes the current compendia culture media as potentially suboptimal in some cases. For example, the FTM aerobic incubation may be unfavorable for strict and facultative anaerobes and fungal and bacterial spore germination and growth. Further, FTM composition characteristics such as viscosity, low redox potential and component toxicity could also halt the growth of vegetative bacteria and fungi. Similarly, incubation of TSB at 20–25 °C may be unfavorable for slow growing skin microbiota sourced by personnel intervention [75]. Research is ongoing to find an alternative competitive culture media for enhancing slow growing bacteria that is not appearing in the given testing time [56, 76]. First drawback of the culture media in sterility confirmation is the substance constituents of the media. Generally, culture media composed of nutrients such as energy source, carbon and nitrogen sources, amnio acids, vitamins, minerals and some substances like bile salts, sodium salts, dyes, antioxidants, antiseptics e.g. cetrimide, and the gelling agent agar which is a basic component of solid media [77]. Selective constituents such as antimicrobials and antiseptics are added purposefully to suppress growth of certain bacterial classes in favor of others to grow. However, some media essentials can interfere with microbial growth.

As an example, research findings demonstrated that the bacterial agarose of soil isolates can degrade agar and prevent growth of some mesophilic and extremophiles species [78]. Moreover, autoclaving of culture medium containing agar and phosphate can yield oxidizing agents such as hydrogen peroxide (H_2O_2), which can be initially inhibitive to microbial growth. Interestingly, separate autoclaving of agar and phosphate, mixed right before solidification, showed a distinct growth of uncultivable microbes [79]. Furthermore, increased concentration of agar in culture media decreases the nutrient diffusion, hence, the bacterial reach to nutrients [77]. As a good practice, upon receipt in the laboratory, culture media should be tested for fertility, this step is crucial to ensure the media performance and reliability for bioburden estimation and sterility test results. Media fertility test is performed by incubation with test microorganisms and assessment of microbial growth. The assessment results are important in validation of test methods and confirmation of the media suitability for growth enhancement of microbial contamination including slow growing bacteria.

6.4 Incubation conditions variability

The incubation temperature and time are other important challenges for growth-based test methods. Some guidelines, such as EU Annex 1 [16], did not specify the incubation parameters for environmental monitoring and aseptic processing simulations. Some studies emphasize the use of current standards, in which the incubation temperature of samples using pour plate method is 30–35 °C. This range has been found appropriate for recovering of distinct and large CFU counts, comparing to other ranges and combinations of temperatures, i.e. 25–35 °C [38].

Currently, there is disagreement around conditions to incubate the test sample separately in different ranges of temperatures for the test period, or together in two subsequent incubation temperatures. This controversy is driven by the anticipated contamination type expected in the manufacturing facility. According to the harmonized (EP) Chap. 2.6.1 [80], the sterility test samples should be incubated separately

at 20–25 °C for TSB and 30–35 °C for FTB for at least for 14 days to allow for broad recovery of fungi and bacteria. In contrast to these conditions, and in order to enhance microbial growth of human contaminants (e.g. Staphylococcus) of aseptic products, the USP recommends two subsequent incubations of the same tested samples, the first at 30–35 °C and the second at 20–25 °C, for 14 days in total. For terminally sterilized items, the incubation for 7 days can be sufficient if the test is validated via membrane filtration.

Similarly, in bioburden estimation, dual subsequent incubations of 2 days at 30–35 °C and 3 days at 20–25 °C is a choice of some manufacturers instead of single 5 days incubation at 30–35 °C. Collectively, single incubation or subsequent two incubations is differently endorsed by guidance documents [81]. Regardless of standards technical divergence towards incubation conditions and time, both EU and USP methodologies aiming at maximize microbial recovery. Generally, standard agar plate technique and laboratory incubation conditions can significantly affect particular enumeration of specified microorganisms [73].

6.5 Erroneous assessment of microbial growth

Counting of bioburden CFU on agar plates is often performed visually with the aid of magnifier and electronic pen for marking grown colonies. This manual counting of CFU is tedious, time-consuming and subject to bioburden data variations and errors [82]. Likewise, visual examination of broth turbidity and colony morphology on Gram's stain slides are prone to inter-individual visual acuity variations [83]. Visual counting errors result from agar plates with heterogenous distribution of large number of colonies e.g. >300 CFU with different diameters and morphologies, or when mold colonies grow on the plate edge, spread and sporulate new colonies which initially not exist in the original sample. Also, when different colonies merged [82, 84], or swarmed such as with *Proteus mirablis* and *Bacillus cereus* bacteria [42]. In addition, counting a tiny colorless colony is visibly difficult on transparent agar plates, especially if the background or brightness of light is not correctly fitted. Moreover, colonies embedded in agar medium become invisible in case of pour plate method is used; particularly with dark background color media such as blood agar [76, 84]. Furthermore, artefacts in agar plate are frequently mistaken for microbial colony [83]. In a study performed on over 13,000 agar plates, to assess the accuracy of counting among operators of three different laboratories, nearly, 1000 plates were read by each operator to evaluate over-(false-positive) and under-counting (false-negative). The results showed that there were 50% variations between operators, mostly with over-counting. Most errors were about difficulties of counting small colonies (range: 53–63 µm) comparing to larger ones (range: 425 and 500 µm). The counting errors were clearly influenced by the position of the colony on the plate and colony contrast, as counting light colonies is more challenging than the darker ones [82, 85]. Counting variations among operators creates a divergence in bioburden results and affects the integrity of analysis data. For eliminating the regulatory concerns around plate count limitations and inter-personnel variations, the US FDA data integrity document recommended two independent operators for bioburden counting data acquisition, namely "double plate counting" or secondary analyst confirmation [85–87].

Automated colony counting is an efficient choice to overcome challenges of data reliability, which arises from erroneous visual counting. The ideal automated system is featured by the capability of capturing colonies of different shapes and sizes, including tiny

CFU in solid or liquid media, and transforming the captured counts to digital format [42]. Different system varieties of commercial brands are available in the market, these systems generate competent results for counting crowded colonies on different agar media including dark colored. But some have been limited by the inability to recognize dust and scratches for bacterial colonies and have concerns of validation and qualification [42, 84]. Difficulties in CFU counting either by visual or automated methods, result in errors and subsequently data robustness issues and false results. Collectively, growth-based microbiological methods encounter lots of variability, inconsistency and bias. Because it is carried by alternate operator personnel, it is subject to different types of errors in sampling, dilution, plating, incubation and result assessment.

6.6 Traditional sterility test limitations

The present argument of inability of the sterility test to guarantee sterility is grounded on some limitations of the compendial growth-based methods (Table 2).

One of the most recurrent discussions in the literature is the challenging sterility test long incubation period and its impact on quick product release [21]. Sterility test durability is essential to support the growth of slow-growing bacteria, such as *Cutibacterium acnes*, which is a skin inhabitant and is often responsible for product contamination [93, 94]. Analyzed data of ten years testing reported that sterility test sample incubation for seven days is insufficient to enhance detection efficacy [95]. Bacterial survivals in an aseptic environment is also slow growing and requires a long time to recover in the culture medium. However, extending the test period over 14 days to encourage the proliferation of slow-growing bacteria might be unsuccessful because they could have converted undetectable microbes to VBNC [66]. In addition, the currently used FTM and TSB liquid media can influence the test results. Published data revealed that only 9% of failed tests showed simultaneous growth in both FTM and TSB. While microbial contamination is supposed to grow in both media, only 55% of positive tests have been observed in TSB and 39% in FTM [75]. These examples demonstrate the downsides of the duration of the compendial sterility test, its microbial specificity, and its ability to detect a low number of microorganisms [17].

Another drawback is the visual assessment of microbial growth on agar or broth medium, which is dependent on incubation time [96]. Broth turbidity becomes visible when the growth density is approximately 10^6 CFU/mL [75], which creates uncertainty about the less intensive growth estimation. Further delay in test results can occur in the case of sterility test failure or microbiological data deviation, this mandates quarantine of product batch and immediate investigation to verify the causes and undertaking of corrective and preventive actions, which imposes further delay [61, 97].

Table 2 Sterility test limitations

Limitation	Description	References
Sample size	Sample size is often not representative of the batch; hence, the test results can be a gross estimate of batch sterility.	[67, 88, 89].
Long duration	Test time takes 14 days to produce results, which is inappropriate for products with short shelf-life.	[21, 90].
Low specificity	Inability to detect, fastidious microorganisms and VBNC microbes, that requires selective media and specific growth conditions.	[91].
False results	Probability to produce false-positive and false-negative results due to cross contamination or improper sample collection and transport.	[22, 92]

Furthermore, the pharmacopeial sterility test encounters additional practical and technical drawbacks related to its execution, which can lead to test failure (Table 3).

The sample size for the sterility test is a debatable issue. The number of test samples is argued as statistically incapable of detecting minor contamination. Field experts' attempts to resolve this issue were unsuccessful because the current compendia are already in line with recognized pharmacopeia. Moreover, regardless of whether the sterility test is the recommended Pharmacopeial test are often mandatory for product release, sterilization professionals considering the sterility test pass decision need not interpret it as absolute sterility of the product batch because of the drawbacks discussed above. In addition, the compendial sterility test could face applicability challenges with novel products. For example, in the testing of patient-specific finished products, which are usually produced in small batches [75]. In addition, the test is incompatible with some novel products with short shelf-life and products for immediate use. These requirements have been considered in the recent USP chapter < 1071>, "Rapid Microbial Tests for Release of Sterile Short-Life Products: A Risk-Based Approach" [101], and the USP chapter < 1223>, "Validation of Alternative Microbiological Methods". The latter discusses the CFU limitations in particular. In addition, The USP chapters < 72>(respiration-based method), <73>(ATP bioluminescence-based), and <74>(solid phase

Table 3 Practical limitations of microbial growth assessment

Limitation	Causes of limitation	Sterility test/ bioburden result	Limitations impacts	Challenges	References
Sampling issues	Sample size inadequate or not batch representative.	False negative	Release of a contaminated batch.	Sample size should resemble the batch size.	[65, 66]
Improper test sample handling.	Test contamination with extraneous non-product related bacteria.	False positive	Reject of a sterile batch.	Operators must appropriately adhere to sterile gloving, gowning and face masking. Outer surface of sample must be disinfected. Testing under strict aseptic conditions. Testing container surface with appropriate method.	[22, 46, 48, 90, 92]
Test sample or culture media containing growth inhibitory agent or a preservative	Presence of bactericidal or fungicidal agents kills the sample bioburden.	False negative	Release of a contaminated batch.	Normalizing samples by chemicals or dilution to inactivate bacteriostatic and fungistatic agents before testing. Culture media should be supplemented with neutralizer and validated before use. Neutralizing agents must be validated for toxicity and efficacy according to USP < 1227>.	[60, 98–100]
Inappropriate culture media used in testing	Low growth promotion capacity of the medium. Solid agar media may prevent nutrient diffusion and halt microbial growth.	False negative	Probability of a non-sterile batch release.	Use of standard quality culture media that fulfill the requirement of ISO 14,698. Particular growth requirement of microorganisms should be taken into account.	[77–79]
Erroneous CFU counting	Personnel variations. Personnel training requirement.	Failure to get accurate bioburden data.	Inaccurate bioburden limit.	Double plate counting by two independent operators (FDA recommendations). Automated colony counting.	[84, 86, 87]

cytometry-based), focuses on rapid microbiological methods for contamination detection in short-life products such as biologicals and cell-based therapies, which are unbearable to the long duration of the traditional sterility test. Limitations in test samples and patient needs are the main reasons for the use of an alternative sterility testing method.

However, until a core change in the sterility test principal, from visible growth of colonies and broth turbidity to a more accurate and specific method, few options remain for improving the current growth-based methodology, for instance, empowering the media recovery conditions through composition change to increase the test sensitivity [89]. Overall, the limitations of growth-based techniques for bioburden and sterility tests are the motive to adopt and regularize rapid sterility testing methods that provide accurate and specific results in an incomparable shorter time.

6.7 Rapid microbiological detection methods

Rapid microbiological methods (RMMs) have been developed for the quick detection of microbial contamination and to address the challenges of reducing the time taken in conventional testing and increasing sensitivity to microbial detection. RMMs are automated systems their potential to improve microbiological quality control is high. RMMs comprise growth-dependent and growth-independent methods [70], both of which can be used effectively in the identification and quantification of microbial contaminants. Growth-based methods detect and quantify only proliferative organisms on solid or liquid media by measuring physiological or chemical growth parameters. Non-growth-based methods utilize technologies such as nucleic acid-based methods [18, 102], growth metabolic by-products [103], mass spectrometry [18], flow cytometry [104–106] and others [107]. These methods can also be classified as qualitative (assessing the microbiological quality as absence of microorganisms) and quantitative (provides numerical result of the microbial content). Noteworthy, the result of the qualitative category of "no growth detected" does not signify an absolute absence of microorganisms. Based on the current compendia, no growth was detected under the applied test conditions. Therefore, the inherent analytical dogma of the compendial methods should be considered when choosing the candidate RMM method [108].

The test-to-result time of RMMs are claimed to range from a few minutes to 5 days, and the best detection sensitivity is about ≤ 1 CFU, which is far less than that of the compendial methods (Table 4). RMMs can support or substitute the compendial pharmacopeial methods and the traditional biochemical and phenotypic identification techniques. Thus, can be employed in quantifying the bioburden as early as in the analysis of raw materials, and water for injection before mixing with raw material. Using RMMs is particularly significant at this phase of manufacturing, allowing for corrective and prevention action (CAPA) to occur in case of contamination; however, the time-to-result using traditional testing is 3–5 days, which means that the product is already packaged by this period [109]. RMMs can be applied in pre-filtration sterilization and aseptic filling and can significantly provide results while the manufacturing process continues. Likewise, implementing non-conformance or CAPA investigations are possible if the RMM results are positive.

Other applications of RMMs in the pharmaceutical manufacturing process include finished non-sterile products, environmental monitoring, in-process bioburden testing, sterility testing of finished products, and identification of microorganisms. Nevertheless,

Table 4 Examples of established and emerging rapid Microbiological detection methods for bioburden and sterility testing

Method	Principal	Application/ test to result time	Pros	Cons	References
Growth-independent methods					
PCR	Microbial nucleic acid amplification	Environmental samples 4–5 h.	Detection sensitivity of bacterial cells: 10–100 CFU. Real time qualitative and quantitative analysis.	Targeted test, confirms or dismiss the existence of a particular microorganism. Possibility of false negative results due to detection limitations of a specific DNA sequence e.g. mutated variant. Possibility of false positive due to contamination by DNA. Inability to discriminate between dead and viable cells. Can be affected by DNA purification, extraction methods and PCR inhibitors. Not suitable for routine work.	[107, 117, 120]
NGS, also known as high-throughput sequencing	Sequencing the genomic libraries of fragmented DNA or RNA strands.	Sterility testing 2 days.	Can simultaneously sequence thousands to billions of DNA fragments independently. Short detection time. Unbiased detection and identification of microorganisms. High sensitivity (0.1 CFU in supernatant or 1 CFU in cell suspension). No need of cell culturing.	Sequencing should be preceded by genetic material amplification. Subject to analysis error, e.g. base error and sequencing bias.	[102]
ScanRDI® System	Solid phase cytometry. Detection is based on capturing dye labeled viable microbes on membrane filter, while the tested drug is passing through.	Sterility testing. 4 h	FDA accepted as an alternative to the conventional sterility test approach. High sensitivity, can detect one viable microbial cell. Ability to detect VBNC. No incubation required, microbial growth or multiplication is not needed.	For filtrable drug products.	[104]

Table 4 (continued)

Method	Principal	Application/ test to result time	Pros	Cons	References
Flow cytometry	Laser scanning cytometry of individual cells basing on shape, size and fluorescent properties.	Sterility test. Bioburden 2 h	Detects VBNC. Single microorganism detection in liquid, so sample incubation is not needed. Provides fast and accurate data. Improvised brands are accurate and faster than 16 S rRNA gene sequencing. Real-time monitoring for bioburden in water.	Conventional fluorescent-based flow cytometry has limited detection channels. Mass cytometry is a cell destructive technique. Unreliable for species recognition.	[18, 105, 106, 109, 118, 121]
Growth-dependent methods					
Milliflex system	Detection is based on ATP bioluminescence of growing microorganisms.	In-process bioburden testing. 5 days	EMA and MHRA approved. Similar to traditional sterility test, so can be easily validated. Membrane filtration technique is accessible and adjustable to sterility testing. Ability of 1 CFU detection. Partially destructive test, leaves large fraction of the colonies for further identification. Can to some extent be useful for quantifying the intensity of contamination. Provides robust and reliable results. Similar to the traditional sterility test in terms of repeatability, limit of detection, specificity and accuracy.	Uses only filterable test samples.	[96, 109]
Celsis Advance System	Detection is based on ATP bioluminescence of growing microorganisms.	Sterility test, in-process, bioburden, and microbial limits. 2 days for microbial limits to 6 days for sterility testing.	The system is usable for filtrable and non-filterable drug products. The system is compatible with various types of pharmaceutical products. Compatible with both direct inoculation and membrane filtration. Similar to conventional sterility test, so can be easily validated. Only small fraction of the incubated volume is needed for the test, giving the chance to use the rest for the identification of the contaminants.	Because of the system requirements, the test is applicable for only non-sterile drug products which is not often contain growing microorganisms. Sample incubation is required using liquid nutrient culture medium.	[96, 109]

Table 4 (continued)

Method	Principal	Application/ test to result time	Pros	Cons	References
Millipore Milliflex Quantum	ATP bioluminescence technology		Can potentially reduce the time to result by several days. Less expensive. Less complicated.	Validation takes long time. Readings are performed manually, and staining steps are laborious and takes longer time. Endorsed by the Code of Federal Regulations Title 21, Sect. 610.12 (21 CFR 610.12) and USP < 71 >.	[62, 96]
BacT/Alert® system	CO_2 monitoring systems		Low cost. Offers several culture media developed for the system. Reduced incubation time.	Have challenges for mold detection. Endorsed by the Code of Federal Regulations Title 21, Sect. 610.12 (21 CFR 610.12) and USP < 71 >.	[62, 112]
Vitek 2 bacteriological analyzer	Fluorescence-based technology	Bioburden identification / 3 h	Database-based identification	Identification accuracy: 42.5–94% There is a chance of error in microbial genus and species identification, as some bacterial species profiles are not existing in the analyzer database. Error at the species level: 6.24%.	[122, 124]
Endospore Germinability Assay (EGA)		Surfaces-Environmental monitoring. Results attainable in minutes.	Bioindicator for bioburden reduction on surfaces. Quantitative technique. Faster than the traditional methods e.g. swab and rinse sampling technique and standard heterotrophic plate count, for surface sterilization validation. Cost effective. Convenient for evaluating bioburden level on surfaces. Efficient in evaluation of decontamination protocols.	Time scale allocated for the EGA test may not allowing some not readily germinable environmental endospore species. May require a well characterized bioindicator spores. Requires more validation on different sterilization protocols and different environments.	[73]

different methods may produce varying results because some systems are more advanced than others, despite that the characteristics of each system can determine the purpose of its use. Nucleic acid-based systems such as polymerase chain reaction (PCR) and next-generation sequencing (NGS) technologies can be used to obtain rapid and accurate real-time results. These growth-independent approaches have efficient sensibility

and capacity as quantitative tests, but they have the drawbacks of primer specificity. While growth-based RMMs such as BACTEC (Becton Dickinson, USA), BacT/ALERT (Biomerieux, France), and VersaTREK (Thermo Fisher Scientific, USA) [103] are comparably slower and less sensitive than other methods, their preference stems from their ease of applicability and similarity to traditional methods in terms of microbial growth and incubation. As an example of system variability, Millipore's Milliflex system is an approved method that uses the detection principle of ATP bioluminescence. The advantages of this test are as follows: the test is growth-based, it has access to membrane filtration technology and can be tailored for sterility testing and similar to conventional sterility testing. Furthermore, the test can detect 1 CFU and provide results in 5 days [96, 110]. The challenge with this application was the validation of the medium used for culture. Nevertheless, a solid nutrient medium suitable for promoting the growth of stressed microorganisms was sought [111]. A study comparing RMMs with conventional methods showed that a growth-dependent BacT/Alert system incubated at 32 °C was equivalent to a direct inoculation USP <71> "Sterility Test" [112]. In contrast, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) has demonstrated higher reliability than total colony counts in predicting contamination of environmental samples [113]. In addition, the results of solid-phase cytometric analysis (single-cell counting) are similar to CFU traditional counting methods, as the colonies counted undoubtedly originate from a single microbial cell, but the interpretation of these results can lead to differences in recovery rates [109].

RMMs have been available for nearly three decades and have been applied in clinical settings and in the food industry. Some RMMs have been in use for sterility testing since nearly two decades ago [96, 110]. Practically, RMM implementation in the pharmaceutical industry has been more hesitant because of the regulatory constraints [114, 115]. However, USP < 1223 > and the EP 5.1.6 acknowledge the use of RMMs [108] as an alternative method for drug sterility testing [103]. USP chapters < 72 > and < 73 > also supporting the incorporation of RMMs advancements in contamination detection which become an essential requirement for the development of quality control methods.

Implementing these methods in the pharmaceutical industry has several challenges, such as the need for high-level technical expertise, experience for accurate performance, installation and running costs, regulatory support, and validation of results [115]. Risk assessment is also required, especially for RMMs capable of detecting VBNC [96, 116]. Validation in particular, comprises extensive testing steps for each product to meet "method suitability testing" requirements of the selected RMM method because there is no single method applicable to all types of medical and pharmaceutical products [117, 118]. Therefore, to facilitate the adoption of RMMs by the pharmaceutical sector and quality organizations, the technical report N. 33 provided by the Parenteral Drug Association (PDA), is a comprehensive guide that offers a holistic approach for the effective assessment and validation process to ensure suitability of RMMs [115, 119].

In addition, regulatory review is required to ensure that the obtained results are accurate and reproducible [75]. However, the use of RMMs and their applications are encouraged by EP and USP. Some multinational companies have gained the FDA, EMA, Medicines and Healthcare Products Regulatory Agency (MHRA), or Japanese Pharmaceuticals and Medical Device Agency (PMDA) approvals for rapid sterility testing [96]. Manufacturers have several RMM systems to choose from for specific applications.

6.8 Future prospective

The use of elaborated compendial bioburden and sterility testing alone have shown deficiency in contamination identification in pharmaceutical products. Defaulting to the requirement for these traditional test methods in standards and regulations can delay the advancement in the new therapeutics in the current era, which must critically possess a sufficient level of sterility assurance. The present sterility assurance regulations may affect the market supply and preclude the rapid adoption of advanced pharmaceutical products [17]. RMMs can quickly identify and quantify product bioburden or microbiological risks as part of contamination control strategies with high specificity and accuracy. The RMMs characteristics outweigh those of growth-based methods and hold a future promise in improvement of the sterility assurance. However, their application in the large-scale manufacturing of pharmaceutical products remains lagging because of the high expenses, the expertise, and the regulatory restrictions.

Nevertheless, in order to increase confidence in the sterility or safety of a product, an integrated component strategy including all possible means of sterility assurance is supported. This can be achieved, on the one hand, by implementing an integrated system that includes applied quality systems, environmental monitoring data on the number of microorganisms in the process and the frequency of contamination obtained through air, water, surface and human sampling. On the other hand, simultaneous improvements are needed to overcome existing sterility assurance challenges. The requirement for a paradigm change in sterility verification involves a new shift towards risk management and the implementation of RMMs and the adoption of process automation to reduce human intervention [17].

7 Conclusion

Based on literature reviewed here, growth-based microbiological methods by itself cannot unequivocally determine the absolute absence of viable of microorganism. The bioburden estimation methods using the methodology described in the current compendial guidelines are only considered estimation methods to reflect the actual microbiological quality of pharmaceutical production. The sampling limitations, testing inaccuracy, test-to-result time and inefficiencies in microbial detection can lead to erroneous judgments about microbial limits and the absence of significant sources of bioburden in the manufacturing process, which can result in health and financial consequences. However, a strategy involving RMMs can provide a highly sensitive system, which can be used to verify the absence of microbial contaminants in a wide variety of samples. It can also verify that the tested samples are free of microbial contamination, VBNC and negligible counts of microbial contaminants in a short period of time. As these methods continue to develop, a future challenge for pharmaceutical manufacturers will be to adopt RMMs to enhance sterility assurance.

Acknowledgements
Not applicable.

Author contributions
NMM conceptualization and writing of the original draft. SSS and KAA article writing and reviewing. Manuscript revised and edited by NMM. All authors read and approved the final manuscript.

Funding
No funds, grants, or other support was received.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Clinical trial number
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 14 April 2025 / Accepted: 21 August 2025
Published online: 27 August 2025

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