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## Highlights

- The use of hygienic perspective improves the evaluation of objectionable microorganisms
- The decision tree alone helps the correct decisions for human health protection
- The suggested approach allows an evaluation based on updated and easily checking information.

# Objectionable microorganisms in pharmaceutical production: validation of a decision tree

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**Abstract**

The release of quality, safe, and effective non-sterile drugs needs to exclude the presence of objectionable microorganisms, which include microorganisms potentially involved in product degradation, or considered as poor hygiene indicator during manufacturing, or causing adverse effect on patient's health. In this paper, a method allowing objective and verifiable evaluations has been investigated through the development of a suitable decision tree with a template for data collection.

The decision tree has been used to establish which microorganisms were objectionables, using several hypothetical scenarios in which 24 different biological agents, both harmless microorganisms and opportunistic pathogens, were combined with 9 different products, representing each type of administration route for non-sterile drugs. The results showed that the use of aforementioned approach makes the microorganisms evaluation easy and verifiable and highlighted that even the microbes initially considered harmless could be objectionable.

**Keywords**: Objectionable microorganism, decision Tree, non-sterile drugs, microbial contamination, drug product quality, Quantitative Microbial Risk Assessment.

## 1. Introduction

The quality, safe and, when applicable, efficacy of products intended for human use (i.e., pharmaceuticals, waters, foods and beverages, cosmetics, antiseptics, and medical devices) are requirements to be guaranteed for placing them on the market, as reported in several European directives (European Commission, 2001, 2004, 2009, 2011). The fulfilment of these requirements is obtained through well-designed, validated, maintained and controlled processes, systems and environments as well as scrupulous observance of Good Manufacturing Practices (GMP), hygiene standards and continuous training of the personnel involved. The microbiological characteristics are essential to assure quality and security of the products intended for human consumption and are specifically regulated (European Commission, 2005; Unites States Pharmacopeia, 2021; Deyer et al., 2004; European Pharmacopeia, 2020). Unfortunately, despite the aforementioned controls, some microorganism (hereafter MO) surviving in non-sterile products could grow later and consequently compromise them and/or cause infections to consumers. Such MOs are called objectionables (Sutton, 2012). The microbiological tests prescribed by the rules governing the release of not-sterile products should contribute to maintain the process under control and capable of giving products free from any reasonable possibility of spoilage and/or to cause infections. However, such tests are minimum requirements and should be combined with a risk assessment of the recovered MOs which do not belong to avoided taxa, in order to evaluate if they represent a risk for quality, security, and efficacy (i.e., they are not frank pathogens or objectionables) (US Food Drugs Administration 2020; Australian Government, 2008). Such evaluation needs a risk-based strategy for the characterization of MOs which could be isolated from products intended for human consumption and a tool for providing clear, documentable, and verifiable decisions. Indeed, when a MO is isolated from a product, the decision on its acceptability should be reviewed and approved before the release and could be verified during an audit. The risk assessment allows classification or quantification of risks derived from the exposure to biological agents based on their impact on human health.

Moreover, the risk assessment can be carried out according to various approaches with different complexity; among them, the more detailed and evidence-based risk assessment approach is represented by Quantitative Microbial Risk Assessment (QMRA) (Haas et al., 2014). The QMRA has been developed over the last two decades and it combines scientific knowledge about the presence and type of MOs, their potential fate, the human exposure, and the health effects. However, in general, the risk assessment should be as simple as possible, finding the right balance between more detailed and evidence-based framework and the usage of assumptions and expert

judgement (World Health Organization, 2016; PDA, 2014; Carducci et al., 2018; Federigi et al., 2020).

Several methods are suggested to evaluate the risks associated to a MO recovered from a pharmaceutical product, especially if it is intended for particular recipients (i.e., immunocompromised patients), from methods based on objective numerical data to those in which subjective ranking are used (Sutton and Jimenez, 2012; Manu-Tawiat et al., 2001). In this context, the use of a decision tree, supplemented by a module to collect the data necessary for the evaluation of the MO, seems the be the most feasible on the basis of manufacturers' needs (World Health Organization, 2016; PDA, 2014). Regardless of the applied methodology, it should be clearly described by a procedure and carefully verified in order to minimize the probability of rejecting acceptable lots or accepting defective ones.

The aim of our work was to develop a decision tree easily implementable and aimed at prompt intervention decisions and verification operations. Moreover, we provide a template to standardize the data search for making decisions. Finally, we applied both tools (decision tree and template) in order to evaluate their ability to assess if a MO isolated from a medicinal product is objectionable (or not).

## 2. Materials and methods

In order to evaluate if a MO recovered from a product intended for human consumption is objectionable or not, the following three fundamental elements were clearly defined:

- The data sheet used to record all the data concerning the MO and the product from which it was isolated.
- The search procedure for the aforementioned data from authoritative bibliographic sources.
- The decision tree to evaluate the MO.

Moreover, the procedure involving the use of these elements was challenged by assuming the recovery of MOs, representative of different sources of contamination and having different virulence, from products with different administration routes.

### *2.1 Data sheet*

Several documents list the main factors to consider in determining if a MO is objectionable or not (Unites States Pharmacopeia, 2021, European Pharmacopeia, 2020; PDA, 2014). The used data sheet included fixed fields (i.e., data, bibliographic or website sources) shown in the following table (Table 1). An extract of the template of the data sheet is reported in the Supplementary information (Figure S1).

***Table 1.*** *Fields to include in data sheet for the evaluation of the MOs*

| Microbe related factors | Product related factors |
|---|---|
| Recent synonyms of the species | Dosage form and chemical-physical characteristics |
| Features, ecology, and habitat | Administration route |
| Diseases due to infection and main sequelae | Susceptibility to spoilage |
| Resistance to antibiotics | Recipients and their susceptibility to infections |
| Resistance to disinfectants, heat and drying | Level of bioburden |
| Main virulence factors | |
| Outbreaks | |
| Recalls | |
| Spoilage due to proliferation | |

### *2.2 Search procedure*

The search procedure included at least: (i) authoritative sources on detailed information on the MO, (ii) institutional databases containing information on the recalls from the market of products intended for human consumption due to microbial contamination, and (iii) journal databases. Data on each evaluated MO were systematically derived from the following books: "Bergey's manual of systematic bacteriology" (Garrity et al., 2009; Vos et al., 2009), "Descriptions of medical fungi" (Kidd et al., 2016), "The microbiological quality of food, foodborne spoilers" (Bevilacqua et al., 2016) and "Disinfection sterilization and preservation" (Block, 2001).

The recalls from the market were collected from the Food and Drug Administration webpage available at https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts. Such database was consulted by searching for the MO, but without selecting any product type in order to embrace drugs, medical devices, and cosmetics.

As journal database we used "Pubmed", available at https://www.ncbi.nlm.nih.gov/pubmed/, performing advanced searches using the following parameters on the field Title/Abstract:

- The official name of the microbial species.
- Pre-established keywords such as "disease", "outbreak", "virulence", and "antibiotic resistance".

When the obtained papers were not exhaustive, we used less generic keywords (for example we replace "disease" with "bacteremia", "pneumonia", or "sepsis").

### *2.3 Decision tree*

The decision tree is a graphical tool often used to choose, through a logical sequence of pre-established questions, if something has (or not) a certain characteristic and is often used in risk analysis (World Health Organization, 2016). In particular, the use of a decision tree has recently been suggested by the Parenteral Drug Association (PDA) to evaluate the objectionable MOs adopting criteria already proposed by this document (e.g., water activity values that prevents the growth of MOs) (PDA, 2014). In the present study, we prepared the tree previously provided by PDA (*Figures 1-4*) in order either to evaluate objectionable MOs and to develop decision-making tool, which are compatible with a systematic assessment, and quick-easy to use for the verification of the choices/decisions. The PDA decision tree considers the current definition of "objectionable", which includes both product-related and recipient-related objectionable MOs, defined as microbes that could unacceptably compromise the quality of the product as well as microbes that could represent an unacceptable risk to consumer health (Sutton, 2012; PDA, 2014). We considered the decision tree of PDA as a reference, but we decided to include a third category to avoid that MOs which are indicator of poor hygiene could wrongly not be taken into consideration, hereafter named "hygiene-related" objectionable MO. Moreover, we tried to improve the reference PDA decision tree making it easier to be followed by users (i.e., Quality Unit) and to be verified during audit/inspections (i.e., U.S. Food and Drug Administration).

***Figure 1.*** *Decision tree flowchart - Start of evaluation: MO isolation. The asterisk indicates that the detected MO is a "specified MO", whose presence is not allowed for such drug (EP chapt. <5.1.4>, USP chapt.<1111>)*

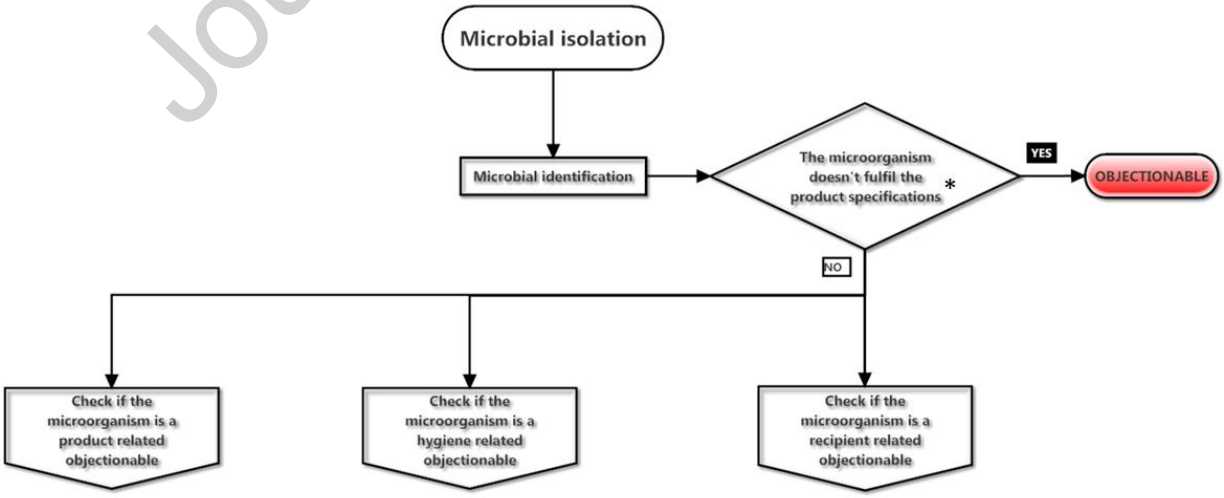


***Figure 2.*** *Decision tree flowchart - Evaluation: Is the MO product-related objectionable?*

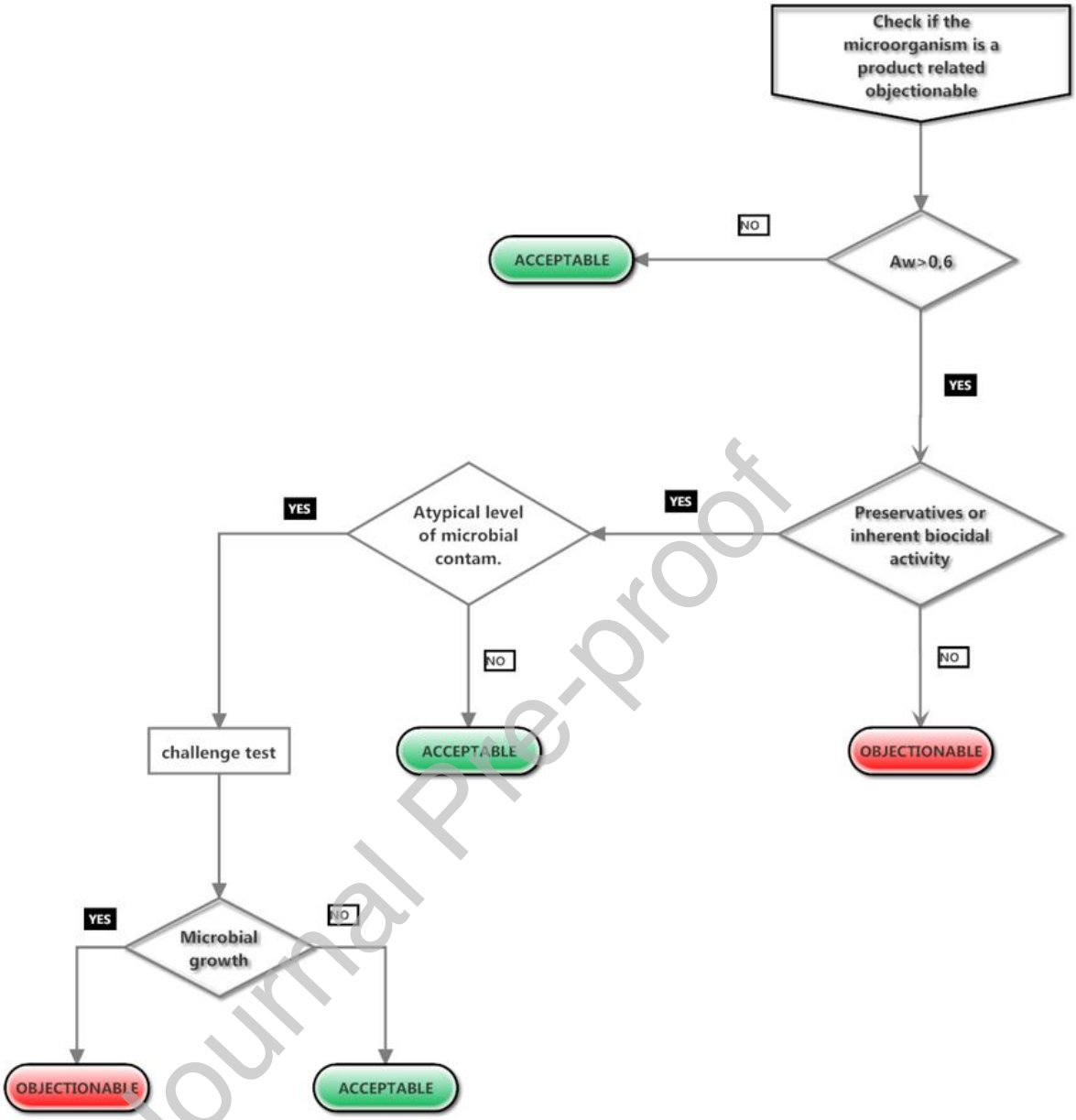


***Figure 3.*** *Decision tree flowchart - Evaluation: Is the MO hygiene-related objectionable?*

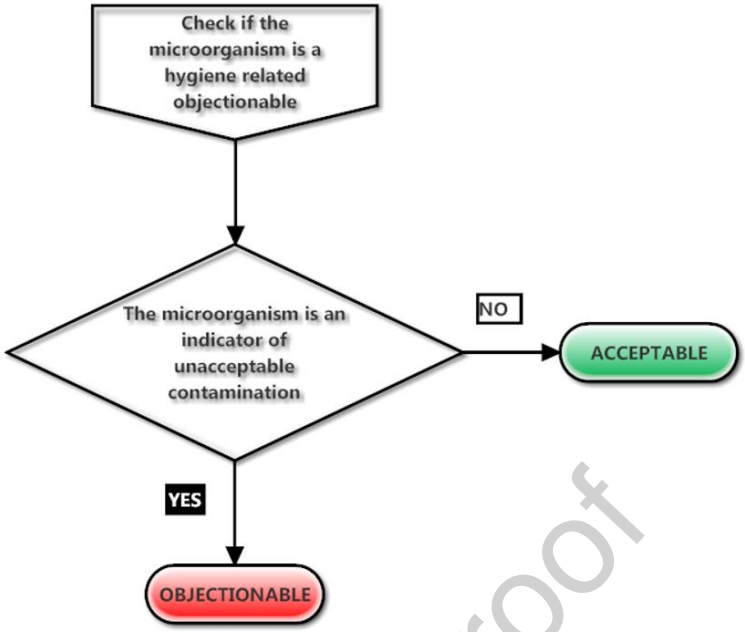
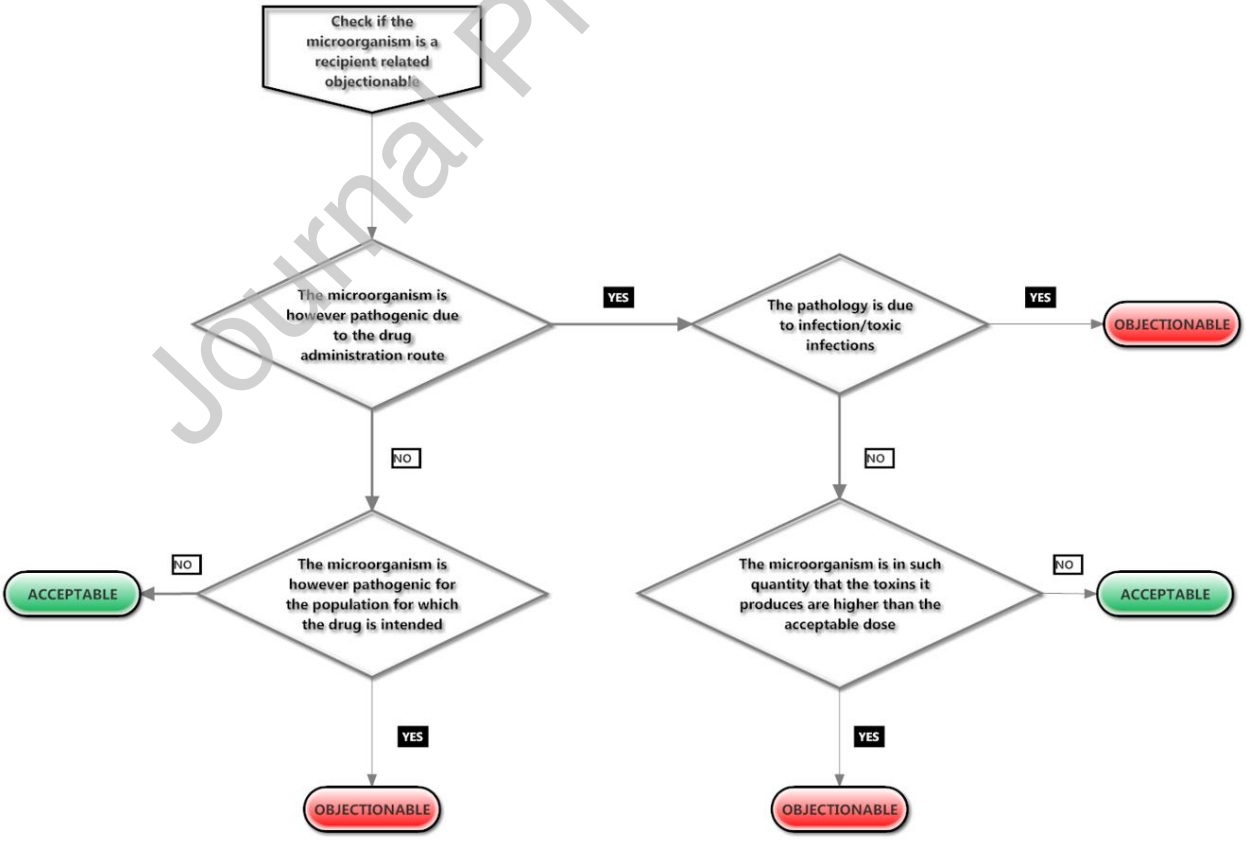



***Figure 4.*** *Decision tree flowchart - Evaluation: Is the MO recipient-related objectionable?*



### *2.4 Chosen MOs*

The "objectionable" assessment procedure (*Table 2* and *Figures 5-7*) has been verified through the definition of a wide spectrum of heterotrophic aerobic mesophilic MOs belonging to different taxa, which include the main MOs involved in recalls from the market (Sutton and Jimenez, 2012), emerging pathogens, environmental isolates and those that are probably harmless.

An emerging pathogen can be defined as a MO that has newly appeared or is rapidly increasing in disease incidence or geographical area. Relations between the pathogen, the host and the environment are critical in determining the emergence of pathogens. In the last years, medical settings facilitated the apparition of multidrug-resistant species (i.e., methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci) that can be considered "emerging pathogens" because of their rapid dissemination among hospitalized patients and the general population, requiring significant attention. However, emerging pathogens can be considered also harmless MOs, normal residents of the skin and mucosa that can infect patients with impaired immune system eliciting atypical syndromes (Vouga and Greub, 2016).

***Table 2.*** *MOs chosen in the evaluation*

| MOs considered | Phylum | Reason of the choice |
|---|---|---|
| *Achromobacter xylosoxidans* | *Proteobacteria* (β) | **a, d** |
| *Acinetobacter baumannii* | *Proteobacteria* (γ) | **c, d** |
| *Alternaria alternata* | *Basidiomycota* | **c** |
| *Bacillus cereus* | *Firmicutes* | **a, c** |
| *Burkholderia cepacia* | *Proteobacteria* (β) | **a, c, d,** |
| *Candida lipolytica* | *Ascomycota* | **c, d** |
| *Corynebacterium minutissimum* | *Actinobacteria* | **b, c** |
| *Cryptococcus neoformans* | *Basidiomycota* | **d** |
| *Elizabethkingia meningoseptica* | *Bacterioidetes* | **a, d** |
| *Enterobacter sakazakii* | *Proteobacteria* (γ) | **a, c, d** |
| *Enterococcus faecalis* | *Firmicutes* | **c, d** |
| *Lactobacillus salivarius* | *Firmicutes* | **b** |
| *Micrococcus luteus* | *Actinobacteria* | **a, c** |
| *Neisseria mucosa* | *Proteobacteria* (β) | **c** |
| *Penicillium citrinum* | *Ascomycota* | **a, c** |
| *Pectobacterium carotovorum* | *Proteobacteria* (γ) | **b** |
| *Pseudomonas aeruginosa* | *Proteobacteria* (γ) | **a, d** |
| *Rhizopus stolonifer* | *Zigomycota* | **c** |
| *Rhodotorula glutinis* | *Basidiomycota* | **d** |
| *Serratia marcescens* | *Proteobacteria* (γ) | **a, d** |
| *Sphingomonas paucimobilis* | *Proteobacteria* (α) | **c, d** |
| *Staphylococcus aureus* | *Firmicutes* | **a, c** |
| *Staphylococcus warnerii* | *Firmicutes* | **a, c** |
| *Streptococcus agalactiae* | *Firmicutes* | **c, d** |

**a)** MO previously involved in recalls
**b)** MO deemed harmless
**c)** MO sometimes recovered from Environmental monitoring (including waters and their purification systems)
**d)** Emerging pathogens

***Figure 5.*** *Phylogenetic relationships of the MOs used to challenge the model (Gram negative bacteria)*

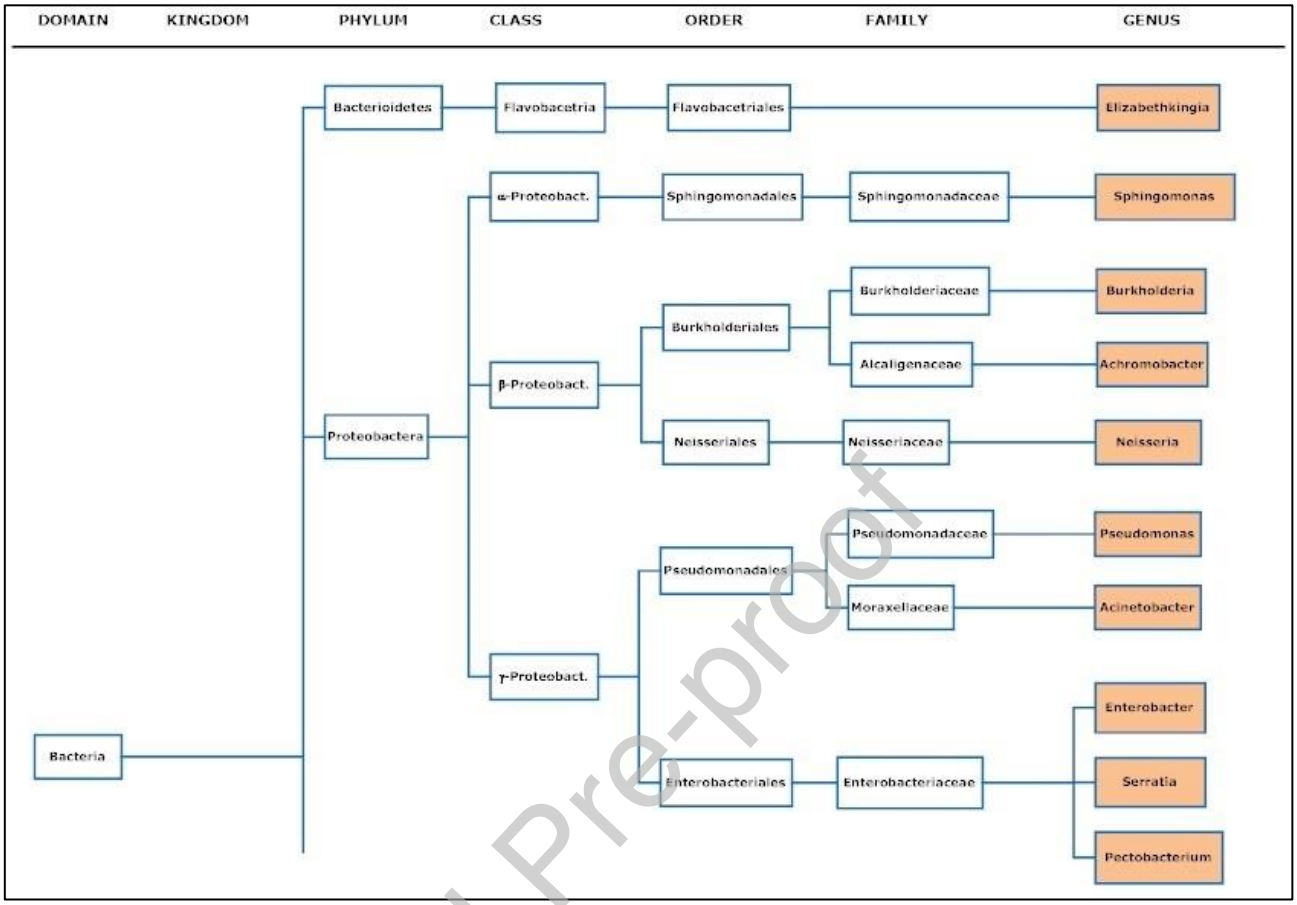


***Figure 6.*** *Phylogenetic relationships of the MOs used to challenge the model (Gram positive bacteria)*

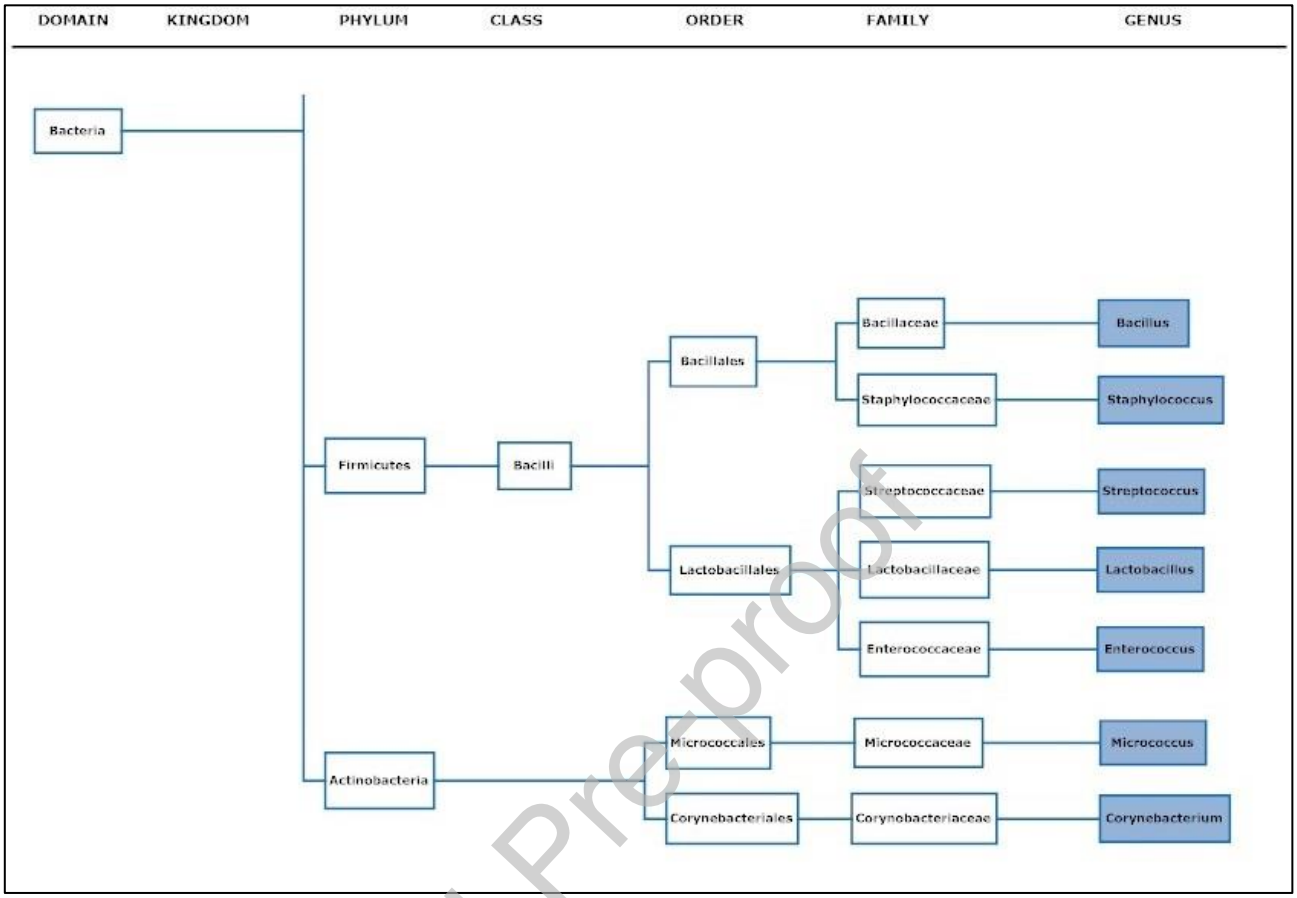
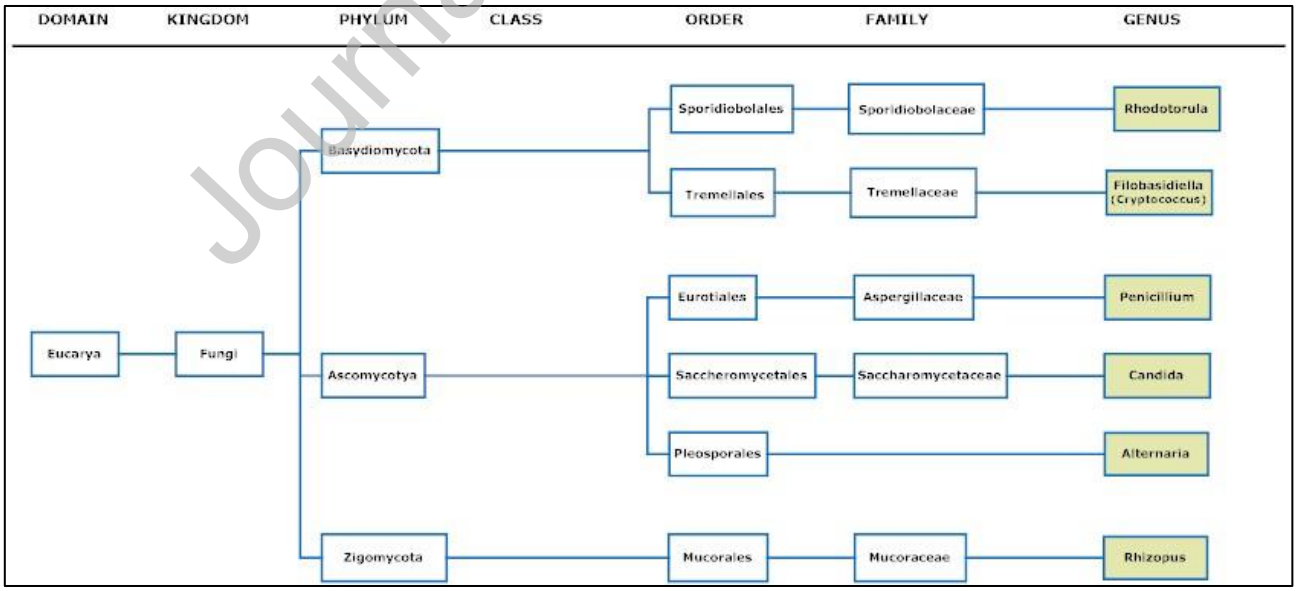



***Figure 7.*** *Phylogenetic relationships of the MOs used to challenge the model (microscopic fungi)*



### *2.5 Products considered*

Various nonsterile medicinal products having different dosage form, route of administration, and target population were hypothesized to have the microbial counts under control and free of any excursion and at meanwhile to harbor the considered MOs (*Table 3*). Information concerning the composition of such products were obtained from the handbook of pharmaceutical manufacturing formulations (Niazi, 2018) and the information concerning administration and target population were obtained from Italian Drug Agency (AIFA) database available at https://farmaci.agenziafarmaco.gov.it/bancadatifarmaci/.

In order to avoid incurring any conflict of interest or violation of rights, we have decided to indicate the aforementioned products with capital letters.

***Table 3.*** *Medicinal products employed in the assessment*

| **Product** | **Dosage Form** | **Route of administr.** | **Target population** | **$A_w$** | **Multidose** | **Preservative** |
|---|---|---|---|---|---|---|
| A | Liquid | Mouth (spray) | All, except newborns | > 0,6 | Yes | Yes |
| B | Liquid | Oral (syrup) | Babies and children | > 0,6 | Yes | Yes |
| C | Liquid | Auricular (drop) | All | > 0,6 | Yes | Yes |
| D | Liquid | Inhalant | All | > 0,6 | No | No |
| E | Semisolid | Rectal | All | ≤ 0,6 | No | No |
| F | Semisolid (gel) | Topical, cutaneous | All, except babies and children | ≤ 0,6 | Yes | Yes |
| G | Semisolid (cream) | Topical | All, except babies and children | ≤ 0,6 | Yes | No |
| H | Semisolid (ointment) | Topical | All, except babies and children | ≤ 0,6 | Yes | No |
| I | Solid | Oral | All, except babies and children | ≤ 0,6 | No | No |

**$A_w$** = Water Activity

## 3. Results

**The following tables (*Tables 4-6*) illustrate the outcome of the performed assessments and show that exceptionally the chosen MOs could be considered free of any risk and that the chosen products, except suppository, are vulnerable to a wide range of MOs. However, the MOs initially deemed harmless were not objectionable for most of the drugs evaluated. Instead, no MO was found to be product-related objectionable because such condition is an unavoidable consequence of the decision tree adopted and the hypothesis of product free of any microbial excursion. Finally, only *Enterococcus faecalis* resulted hygiene-related objectionable because the other chosen MOs could not be considered fecal.**

***Table 4.*** *Results of the assessments using proposed decision tree*

| Product | Gram negative bacteria | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***Elizabethkingia meningoseptica*** | ***Sphingomonas paucimobilis*** | ***Burkholderia cepacia*** | ***Achromobacter xylosoxidans*** | ***Neisseria mucosa*** | ***Pseudomonas aeruginosa*** | ***Acinetobacter baumannii*** | ***Enterobacter sakazakii*** | ***Serratia marcescens*** | ***Pectobacterium carotovorum*** |
| **A** | R | R | R | R | | S | R | R | R | |
| **B** | R | R | R | | | R | | R | R | |
| **C** | R | R | R | R | R | S | R | R | R | |
| **D** | R | R | R | R | | S | R | S | S | S |
| **E** | | | | | | | | | | |
| **F** | R | R | R | R | R | S | R | R | R | |
| **G** | R | R | R | R | R | S | R | R | R | |
| **H** | R | R | R | R | R | S | R | R | R | |
| **I** | | | | | | | | | | |

**Legend**

| **Objectionable** | | **Not Objectionable** | |
|---|---|---|---|
| R | H | P | S |
| (Recipient Related) | (Hygiene Related) | (Product Related) | (Specif. Related) |

***Table 5.*** *Results of the assessments using proposed decision tree*

| Product | Gram positive bacteria | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | ***Bacillus cereus*** | ***Staphylococcus aureus*** | ***Staphylococcus warnerii*** | ***Streptococcus agalactiae*** | ***Lactobacillus salivarius*** | ***Enterococcus faecalis*** | ***Micrococcus luteus*** | ***Corynebacterium minutissimum*** |
| **A** | R | S | | R | | H, R | | |
| **B** | R | R | R | R | | H | | |
| **C** | R | S | R | R | | H, R | R | |
| **D** | R | S | | R | | H, R | | |
| **E** | | | | | | H | | |
| **F** | R | **S** | R | R | | H, R | R | |
| **G** | R | **S** | R | R | | H, R | R | |
| **H** | R | **S** | R | R | | H, R | R | |
| **I** | R | | | R | | H | | |

**Legend**

**Objectionable** **Not Objectionable**

R (Recipient Related) H (Hygiene Related) P (Product Related) S (Specif. Related)

***Table 6.*** *Results of the assessments using proposed decision tree*

| Product | Fungi | | | | | |
|---|---|---|---|---|---|---|
| | ***Rhodotorula glutinis*** | ***Cryptococcus neoformans*** | ***Penicillium citrinum*** | ***Candida lipolytica*** | ***Alternaria alternata*** | ***Rhizopus stolonifer*** |
| **A** | | R | R | | R | R |
| **B** | R | | | | R | R |
| **C** | | R | R | R | R | R |
| **D** | R | R | R | R | R | R |
| **E** | | | | | | |
| **F** | | R | R | R | R | R |
| **G** | | R | R | R | R | R |
| **H** | | R | R | R | R | R |
| **I** | | | | | | |

**Legend**

| **Objectionable** | | **Not Objectionable** | |
|---|---|---|---|
| R | H | P | S |
| (Recipient Related) | (Hygiene Related ) | (Product Related) | (Specif. Related) |

## 4. Discussion

In our assessment, we developed and verified a procedure for a rapid and systematic evaluation of any MO isolated from nonsterile pharmaceuticals, based on authoritative documents and suitable to an easy verification. The methodology included the data search methods and the data sheets for their registration as well as an exhaustive decision tree developed on the basis of Technical Report 67 issued by Parenteral Drug Association (PDA, 2014).

The microbiological laboratories for quality control should be capable to perform quick antimicrobial effectiveness testing to establish the risk of spoilage when the levels of microbial counts are atypical, and the drug is capable of supporting microbial growth (Figure 2).

The choice to evaluate the compliance of MOs also from a hygienic perspective represents an unquestionable improvement, because it implies the rejection of products otherwise considered acceptable and undoubtedly guarantees the microbiological quality (e.g., suppositories contaminated by MOs of likely fecal origin such as *Enterococcus faecalis*).

**Efficiency, speed, and accuracy of the evaluation could be further improved through a suitable software, compliant with the Code of Federal Regulations (US Food and Drug Administration, 2003), allowing the storage of the collected data and their treatment.**

Although the evaluated MOs are opportunistic pathogens belonging to the biosafety levels 1 and 2 (Center for Disease Control and Prevention, 2020), they have been found objectionable for a wide spectrum of products. Such result was expected since the drugs are manufactured products intended to be consumed by people particularly susceptible to infections and the main MOs involved in recalls from the market just belong to the levels 1 and 2 (Sutton and Jimenez, 2012).

However, no MO was product-related objectionable, despite some species can grow in preserved products or in disinfectants. This output derived from our hypothesis that multidose products were not affected by microbial excursions, otherwise the decision tree would still have provided a confirmation challenge.

The outcome of each combination MO/product cannot be extended to other products having the same administration route, because of the differences concerning the target population. Indeed, *Enterobacter sakazkii* in product B (oral syrup for children) is a recipient-related objectionable, but if the product was not for children the outcome would be different. The accuracy of the evaluation carried out with the proposed decision tree can be improved by introducing further steps for example with the aim to assess the severity of infections and their sequelae, anyway the decision tree remains valid as the first screening tool. In fact, the use of the decision tree implies that both *Burkholderia cepacia* and *Sphingomonas paucimibilis* are considered objectionable in cutaneous products, however it is true that the first ones are more virulent (the same reasoning can also be

done for other couples of MOs, such as *Candida lipolytica* vs *Alternaria alternata*). Moreover, *Pectobacterium carotovorum* was evaluated objectivable only in the inhalant route of transmission because it belongs to Enterobacteriaceae family, Gram negative bile tolerant, that is a group not allowed in this type of product by European Pharmacopeia (chapter 5.1.4) and United States Pharmacopeia (chapter <1111>) while *Streptococcus agalactiae, Micrococcus luteus* and *Corynebacterium minutissimum* were differently considered on F-H products (Table 3).

## 5. Conclusion

The current strategy for objectionable exclusion includes two possible approaches. The firm could use the decision tree alone to establish a list of objectivable MOs monitored on a risk basis: such approach allows correct decisions for human health protection, but the MOs list should be frequently updated according to the news scientific knowledges. On the other hand, the second approach relies on the monitoring of bioburden, therefore the detected MOs are evaluated as objectivables from time to time, on the basis of the more recent scientific documents. To decide that a MO is harmless, we need to consider not only its infectivity and biological significance, but also the type of product, the recipients, and the capacity to degrade the drugs. Such body of knowledge is constantly evolving so, in our opinion, the latter strategy represents a more reliable approach. Nevertheless, standardization of monitoring is needed to define a minimum frequency of measurements and the obligations in case of threshold exceeding, as prescribed by PDA Technical Report and United States Pharmacopeia chapter <1115>. Moreover, the chosen approach could be improved with further steps, such as the analysis of the obtained outputs by quantitative methods (i.e., QMRA) and the informatization for enhancing data integrity.



## Credit Authors Statement

Susi Burgalassi: Conceptualization, Methodology, Writing - Original Draft
Stefano Ceccanti: Methodology, Writing - Original Draft, Writing - Review & Editing
Sandra Vecchiani: Writing - Review & Editing
Giulia Leonangeli: Writing - Original Draft
Ileana Federigi: Writing - Original Draft, Writing - Review & Editing
Annalaura Carducci: Writing - Original Draft, Writing - Review & Editing
Marco Verani: Supervision, Writing - Original Draft, Writing - Review & Editing

**Declarations of interest**: none

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