# Ad hoc meeting AMFEP, EUROPABIO and FEFANA

## Guidance on the characterisation of microorganisms

**Brussels, 20 November 2018**

efsa
European Food Safety Authority

# Draft Agenda

1. Welcome
2. Agreement of the draft Agenda
3. Scope of the meeting
4. EFSA: General overview on the Guidance on the characterisation of microorganisms used as feed additives or as production organisms
5. Stakeholders: Presentation of the scientific questions
6. Discussion
7. Summary and conclusions of the meeting

# 3. Scope of this meeting

To tackle a request received by EFSA from AMFEP-EUROPABIO-FEFANA (July 2018) regarding the ‘guidance on the characterization of microorganisms used as feed additives or as production organisms’

- Whole genome sequence as a tool to characterize microorganisms
- MIC threshold values
- Requirements for the absence of rDNA

How? Ad hoc meeting with stakeholders

# About ad hoc meetings

- EFSA Catalogue of Services
- Organised on a case-by-case basis
- Exchange information and views between EFSA and stakeholders on methodological and procedural aspects, scientific requirements or approach(es) which are unique to particular scientific areas and cannot be handled with already available support initiatives
- Direct Communication and an open dialogue

# Participants

- Participants from the industry

| | |
|---|---|
| Philippe Guion, Marc Leclerc (Chair), Alicia Juárez and Markus Wyss | FEFANA |
| Yvonne Agersø, Kees Broekhuizen, Marianne Dessen-Mugniot, Carsten Hjort and Bas Verhagen | AMFEP |
| Agnes Borg, Elke Duwenig, Alexandra Lensch | EuropaBio |

# Participants

- Participants from EFSA

| | |
|---|---|
| Pier Sandro Cocconcelli, Lieve Herman, Baltasar Mayo and Maria Saarela | EFSA experts |
| Manuela Tiramani (Chair) | Head of FEED Unit |
| Montserrat Anguita, Rosella Brozzi and Jaume Galobart | Scientific Officers FEED Unit |
| Jaime Aguilera | Scientific Officer FIP Unit |
| Frank Verdonck | Team leader AHAW |

# Participants

- Participants from EC

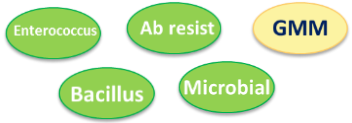
| | |
|---|---|
| Marta Ponghellini, Almudena Rodriguez, Wolfgang Trunk | DG SANTE |

# 4. The guidance document - Timelines

| | |
|---|---|
| May 2016 | • Statement on FEEDAP Guidance documents update plan |
| July 2016 | • Info-session – discussion with stakeholders |
| From September 2016 | • Update started |
| May-September 2017 | • Public consultation (endorsement 18 May) |
| February 2018 | • Adoption on 21 February 2018 – Applicability date 1st of September 2018. |

# 4. The Guidance document – Generalities

- ALL IN ONE document – includes several old guidances

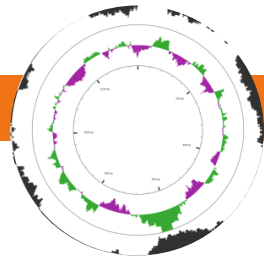



- Some new/updated requirements or new approaches
- Horizontal to all types of additives
- Covers the **characterisation of microbial strains** which are the subject of applications for authorisation of **feed additives** containing or produced with microorganisms (excluding viable GMM)

# 4. The guidance document - Requirements

| | Feed additives containing viable microorganisms | | Fermentation products | |
|---|---|---|---|---|
| | Bacteria | Fungi/yeasts | Bacteria | Fungi/yeasts |
| **Identification** | ✓ | ✓ | ✓ | ✓ |
| **Antimicrobial susceptibility** | ✓ | | ✓ | |
| **Antimicrobial production** | ✓ | ✓ | ✓ | ✓ |
| **Toxigenicity and pathogenicity** | ✓ | ✓ | ✓ | ✓ |
| **Genetic modification** | | | For GMMs | For GMMs |
| **Absence of the production strain** | | | ✓ | ✓ |
| **Presence of DNA from the production strain** | | | where relevant | where relevant |

# WHOLE GENOME SEQUENCE

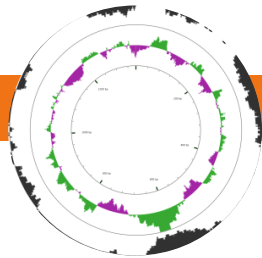


# WGS as a tool to improve the assessment

It provides the most complete genetic dataset for a reliable and affordable identification and characterization

Routinely used for bacteria and yeasts

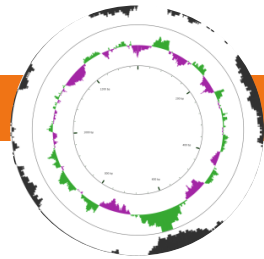
# 4. The Guidance document - WGS



What for:

- Species and strain identification
- Characterisation of genetic modifications
- Search for genes for antibiotic resistance
- Search for genes for toxins/virulence

# 4. The Guidance document - WGS

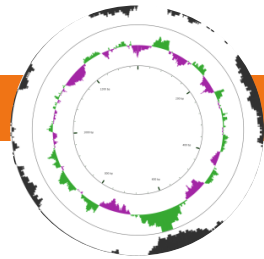


## Species identification

The organism under assessment should be identified unambiguously at species level based on up-to-date methodologies and current knowledge

WGS data **must be** used for the identification of **bacteria and yeasts**, recommended for filamentous fungi.

# 4. The Guidance document - WGS

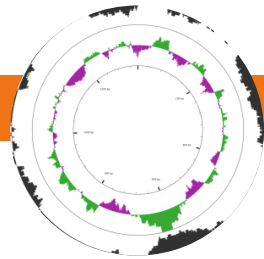


## Species identification

The guidance indicates how to use the WGS data

- *Bacteria:* computational approach for taxonomic assignments, or comparing sequences commonly used for taxonomic identification or other characteristic genes to relevant databases.
- *Yeasts and filamentous fungi:* by phylogenomic analysis

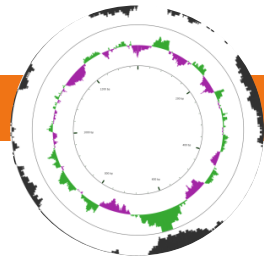
# 4. The Guidance document - WGS



## Genetic modification structure
### *Characterisation*

**Until now,** the applicants described all the steps followed in order to identify all the genetic material potentially introduced.

**From now on,** this must (bacteria and yeasts)/can (for fungi) be addressed comparing the WGS from the GM strain with that of the non-modified parental or recipient strain

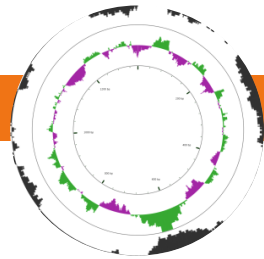


## Genetic modification structure

The guidance indicates what should be presented

- A map/graphic of all genomic regions harbouring genetic modifications indicating the open reading frames and non-coding sequence/s actually inserted, modified or deleted (including genes of concern)
- The sequences/databases and the methodology used for analyses and comparison should be described in detail.

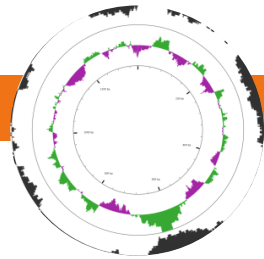
# 4. The Guidance document - WGS



## Search for sequences of concern

This includes genes coding for or contributing to **resistance to antimicrobials** relevant to their use in humans and animals (non-QPS bacteria), **virulence factors** (bacteria), known metabolic pathways involved in **toxigenicity** (eucariota)

# 4. The Guidance document - WGS

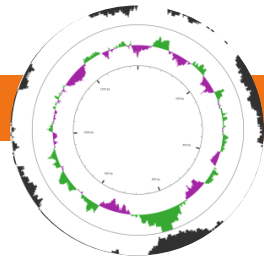


## Search for sequences of concern

**HOW:** comparison of the WGS against up-to-date databases should be performed and outcome be reported

Data not used in isolation, phenotypic testing (compulsory for AMR)

# 4. The Guidance document – WGS



## Reporting requirements for WGS:

- the DNA extraction method
- the sequencing strategy and instrumentation used
- the assembly method applied
- the statistical measure of sequence quality
- the total length of contigs relative to the expected genome size
- the annotation protocol used
- for fungi: information on the quality of the annotations obtained from relevant databases
- the FASTA file(s) of the WGS

# ANTIMICROBIAL RESISTANCE

# Antimicrobial resistance

- Microbial feed additives should not add to the pool of AMR genes already present in the gut bacterial population or otherwise increase the spread of AMR. The antimicrobials considered are those relevant to their use in humans and animals

- Testing applies to all bacteria intended for use as viable cells in feed additives or used as production organisms

# Antimicrobial resistance

INTRINSIC *vs* ACQUIRED

For this, two sets of data should be provided:

- Phenotypic testing based on determination of a minimum inhibitory concentration (MIC) for a selected group of antimicrobials
- A search of the WGS for the presence of known AMR genes

# 4. The Guidance document - AMR

| Experimental tests to find the minimum inhibitory concentrations (MICs) |
|---|
| Ampicillin |
| Vancomycin |
| Gentamycin |
| Kanamycin |
| Streptomycin |
| Erythromycin |
| Clindamycin |
| Tetracycline |
| Chloramphenicol |
| Tylosine |
| Ciprofloxacin |
| Colistine |
| Fosfomycin |

| WGS and search for known antimicrobial resistance genes | |
|---|---|
| Critically Important Antimicrobials | According to WHO |
| Highly Important Antimicrobials | |

| Both approaches are combined | MIC > cut-off | MIC ≤ cut-off |
|---|---|---|
| Gene found | HAZARD | Further studies to determine whether the gene may become active |
| Gene not found | Uncertainty: case-by-case assessment | OK |

# Phenotypic testing

- Minimum inhibitory concentrations (MICs) should be determined for the antimicrobials listed in the Guidance which allow to detect a wide range of resistance determinants.
- The cut-off values provided should be seen as a pragmatic tool intended to distinguish strains with acquired resistance from susceptible strains

# Phenotypic testing

Already present in the previous relevant guidance BUT,

- The cut-off values have been updated
- Some modifications species/groups
- List of antimicrobials updated

# 4. The guidance document - AMR

**Table 2:** Microbiological cut-off values (mg/L)

| | Ampicillin | Vancomycin | Gentamicin | Kanamycin | Streptomycin | Erythromycin | Clindamycin | Tetracycline | Chloramphenicol | Tylosin | Ciprofloxacin | Colistin | Fosfomycin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Lactobacillus*** obligate homofermentative[(a)] | 2 | 2 | 16 | 16 | 16 | 1 | 4 | 4 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus acidophilus*** group | 1 | 2 | 16 | 64 | 16 | 1 | 4 | 4 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus*** obligate heterofermentative[(b)] | 2 | n.r. | 16 | 64 | 64 | 1 | 4 | 8[(c)] | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus reuteri*** | 2 | n.r. | 8 | 64 | 64 | 1 | 4 | 32 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus*** facultative heterofermentative[(d)] | 4 | n.r. | 16 | 64 | 64 | 1 | 4 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus plantarum/pentosus*** | 2 | n.r. | 16 | 64 | n.r. | 1 | 4 | 32 | 8 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus rhamnosus*** | 4 | n.r. | 16 | 64 | 32 | 1 | 4 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus casei/paracasei*** | 4 | n.r. | 32 | 64 | 64 | 1 | 4 | 4 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Bifidobacterium*** | 2 | 2 | 64 | n.r. | 128 | 1 | 1 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Pediococcus*** | 4 | n.r. | 16 | 64 | 64 | 1 | 1 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Leuconostoc*** | 2 | n.r. | 16 | 16 | 64 | 1 | 1 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactococcus lactis*** | 2 | 4 | 32 | 64 | 32 | 1 | 1 | 4 | 8 | n.r. | n.r. | n.r. | n.r. |
| ***Streptococcus thermophilus*** | 2 | 4 | 32 | n.r. | 64 | 2 | 2 | 4 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Bacillus*** | n.r. | 4 | 4 | 8 | 8 | 4 | 4 | 8 | 8 | n.r. | n.r. | n.r. | n.r. |
| ***Propionibacterium*** | 2 | 4 | 64 | 64 | 64 | 0.5 | 0.25 | 2 | 2 | n.r. | n.r. | n.r. | n.r. |
| ***Enterococcus faecium*** | 2 | 4 | 32 | 1,024 | 128 | 4 | 4 | 4 | 16 | 4 | n.r. | n.r. | n.r. |
| ***Corynebacterium* and other Gram-positive** | 1 | 4 | 4 | 16 | 8 | 1 | 4 | 2 | 4 | n.r. | n.r. | n.r. | n.r. |
| **Enterobacteriaceae** | 8 | n.r. | 2 | 8 | 16 | n.r. | n.r. | 8 | n.r. | n.r. | 0.06 | 2 | 8 |

n.r.: not required.
(a): Including *L. delbrueckii, L. helveticus*.
(b): Including *L. fermentum*.
(c): For *L. buchneri* the cut-off for tetracycline is 128.
(d): Including the homofermentative species *L. salivarius*.

# 4. The guidance document - AMR

**Table 2:** Microbiological cut-off values (mg/L)

| | Ampicillin | Vancomycin | Gentamicin | Kanamycin | Streptomycin | Erythromycin | Clindamycin | Tetracycline | Chloramphenicol | Tylosin | Ciprofloxacin | Colistin | Fosfomycin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Lactobacillus*** obligate homofermentative[(a)] | 2 | 2 | 16 | 16 | 16 | 1 | 4 | 4 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus acidophilus*** group | 1 | 2 | 16 | 64 | 16 | 1 | 4 | 4 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus*** obligate heterofermentative[(b)] | 2 | n.r. | 16 | 64 | 64 | 1 | 4 | 8[(c)] | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus reuteri*** | 2 | n.r. | 8 | 64 | 64 | 1 | 4 | 32 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus*** facultative heterofermentative[(d)] | 4 | n.r. | 16 | 64 | 64 | 1 | 4 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus plantarum/pentosus*** | 2 | n.r. | 16 | 64 | n.r. | 1 | 4 | 32 | 8 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus rhamnosus*** | 4 | n.r. | 16 | 64 | 32 | 1 | 4 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus casei/paracasei*** | 4 | n.r. | 32 | 64 | 64 | 1 | 4 | 4 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Bifidobacterium*** | 2 | 2 | 64 | n.r. | 128 | 1 | 1 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Pediococcus*** | 4 | n.r. | 16 | 64 | 64 | 1 | 1 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Leuconostoc*** | 2 | n.r. | 16 | 16 | 64 | 1 | 1 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactococcus lactis*** | 2 | 4 | 32 | 64 | 32 | 1 | 1 | 4 | 8 | n.r. | n.r. | n.r. | n.r. |
| ***Streptococcus thermophilus*** | 2 | 4 | 32 | n.r. | 64 | 2 | 2 | 4 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Bacillus*** | n.r. | 4 | 4 | 8 | 8 | 4 | 4 | 8 | 8 | n.r. | n.r. | n.r. | n.r. |
| ***Propionibacterium*** | 2 | 4 | 64 | 64 | 64 | 0.5 | 0.25 | 2 | 2 | n.r. | n.r. | n.r. | n.r. |
| ***Enterococcus faecium*** | 2 | 4 | 32 | 1,024 | 128 | 4 | 4 | 4 | 16 | 4 | n.r. | n.r. | n.r. |
| ***Corynebacterium* and other Gram-positive** | 1 | 4 | 4 | 16 | 8 | 1 | 4 | 2 | 4 | n.r. | n.r. | n.r. | n.r. |
| **Enterobacteriaceae** | 8 | n.r. | 2 | 8 | 16 | n.r. | n.r. | 8 | n.r. | n.r. | 0.06 | 2 | 8 |

n.r.: not required.
(a): Including *L. delbrueckii, L. helveticus*.
(b): Including *L. fermentum*.
(c): For *L. buchneri* the cut-off for tetracycline is 128.
(d): Including the homofermentative species *L. salivarius*.

# 4. The guidance document - AMR

**Table 2:** Microbiological cut-off values (mg/L)

| | Ampicillin | Vancomycin | Gentamicin | Kanamycin | Streptomycin | Erythromycin | Clindamycin | Tetracycline | Chloramphenicol | Tylosin | Ciprofloxacin | Colistin | Fosfomycin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Lactobacillus*** obligate homofermentative[(a)] | 2 | 2 | 16 | 16 | 16 | 1 | 4 | 4 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus acidophilus*** group | 1 | 2 | 16 | 64 | 16 | 1 | 4 | 4 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus*** obligate heterofermentative[(b)] | 2 | n.r. | 16 | 64 | 64 | 1 | 4 | 8[(c)] | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus reuteri*** | 2 | n.r. | 8 | 64 | 64 | 1 | 4 | 32 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus*** facultative heterofermentative[(d)] | 4 | n.r. | 16 | 64 | 64 | 1 | 4 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus plantarum/pentosus*** | 2 | n.r. | 16 | 64 | n.r. | 1 | 4 | 32 | 8 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus rhamnosus*** | 4 | n.r. | 16 | 64 | 32 | 1 | 4 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactobacillus casei/paracasei*** | 4 | n.r. | 32 | 64 | 64 | 1 | 4 | 4 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Bifidobacterium*** | 2 | 2 | 64 | n.r. | 128 | 1 | 1 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Pediococcus*** | 4 | n.r. | 16 | 64 | 64 | 1 | 1 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Leuconostoc*** | 2 | n.r. | 16 | 16 | 64 | 1 | 1 | 8 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Lactococcus lactis*** | 2 | 4 | 32 | 64 | 32 | 1 | 1 | 4 | 8 | n.r. | n.r. | n.r. | n.r. |
| ***Streptococcus thermophilus*** | 2 | 4 | 32 | n.r. | 64 | 2 | 2 | 4 | 4 | n.r. | n.r. | n.r. | n.r. |
| ***Bacillus*** | n.r. | 4 | 4 | 8 | 8 | 4 | 4 | 8 | 8 | n.r. | n.r. | n.r. | n.r. |
| ***Propionibacterium*** | 2 | 4 | 64 | 64 | 64 | 0.5 | 0.25 | 2 | 2 | n.r. | n.r. | n.r. | n.r. |
| ***Enterococcus faecium*** | 2 | 4 | 32 | 1,024 | 128 | 4 | 4 | 4 | 16 | 4 | n.r. | n.r. | n.r. |
| ***Corynebacterium* and other Gram-positive** | 1 | 4 | 4 | 16 | 8 | 1 | 4 | 2 | 4 | n.r. | n.r. | n.r. | n.r. |
| **Enterobacteriaceae** | 8 | n.r. | 2 | 8 | 16 | n.r. | n.r. | 8 | n.r | n.r. | 0.06 | 2 | 8 |

n.r.: not required.
(a): Including *L. delbrueckii, L. helveticus*.
(b): Including *L. fermentum*.
(c): For *L. buchneri* the cut-off for tetracycline is 128.
(d): Including the homofermentative species *L. salivarius*.

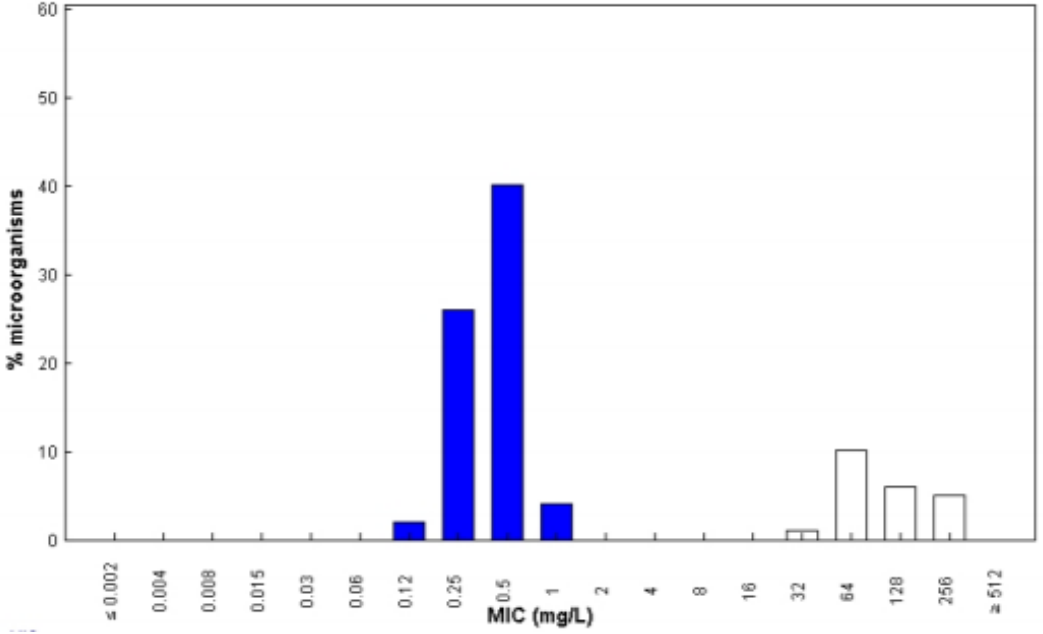
# 4. The guidance document - AMR

- Cut-off values in the guidance were based on current scientific knowledge. Published and official sources were used and the references are given in the guidance.
- A cut-off value can be defined only when the dataset is big enough. This dataset should include **independent** wild-type strains from **different sources**, ensuring a correct distribution of the MIC values.

# 4. The guidance document - AMR

**EUCAST 'Implications of breakpoints splitting the wild type and/or resistant populations**

Tetracycline MIC distributions for *Staphylococcus aureus* (Reference Database)




**MIC epidemiological cut-off (ECOFF):** 1 mg/L

**Wildtype organisms** ≤ 1 mg/L

1879 Observations
(12 data sources)

# 4. The guidance document - AMR

- The current strategy for separating the bacteria is based on the data available.
- The list of microbes given in the Guidance reflects the experience gained so far in assessing microbial feed additives applications and the available body of knowledge.
- Other entries might be considered in the future, if needed.

# PRESENCE OF DNA

# 4. The Guidance document – Presence of DNA

Presence of DNA should be evaluated **in fermentation products** whenever,

- ✓ The production strain is a **genetically modified** production strain, in compliance with regulatory requirements. The presence of the DNA may not be a safety concern (ie., no genes of concern found in strain).
- ✓ For products obtained using **non-genetically modified** production strains carrying acquired AMR genes

# 4. The Guidance document – Presence of DNA

Very common question in dossiers dealing with additives produced with GM strains.

The Guidance details how to perform the PCR analysis:

- Target sequence
- Amount of sample
- Number of batches – replicates to analyse
- Lysis step to ensure detection of trapped DNA
- Sensitivity tests

Establishes threshold of 10 ng/g or mL of product; the LOD has to be lower or equal to 10 ng/g or mL

# 4. The Guidance document – Presence of DNA

## How to perform the analysis – Target

- ✓ If production strain carries AMR genes primers should be designed to amplify a fragment not exceeding the size of the smallest antimicrobial resistance gene.
- ✓ If the production strain is a GMM not containing AMR genes, the targeted sequence should cover maximum 1 Kb

# 4. The Guidance document – Presence of DNA

## How to perform the analysis – Samples

- ✓ Analysis of the additive
  - Upstream intermediate products can be used with in many cases less problems with PCR inhibition
  - Additives with different formulations, use the most concentrated if production scheme is the same otherwise all formulations/products should be tested.
  - Industrial scale/pilot scale
- ✓ At least three independent batches of product analysed in triplicate.
- ✓ DNA to be extracted from > 1 g or mL of product

# 4. The Guidance document – Presence of DNA

## How to perform the analysis – Sensitivity

- ✓ total DNA from the production strain **(+ control PCR)**
- ✓ total DNA from the production strain added to the sample **before** the DNA extraction process **(LOD)**
- ✓ total DNA from the production strain added to the DNA extracted for checking **PCR failure**
- ✓ a negative control without sample

# 4. The Guidance document – Presence of DNA

## How to perform the analysis – Other

- ✓ DNA that may be trapped in non-viable cells should be extracted. A lysis step should follow a methodology suitable for all cellular forms of the production strain (e.g., vegetative cells, spores)
- ✓ if PCR failure is encountered, the causes should be investigated (e.g. PCR inhibition, presence of nucleases)

# 4. The Guidance document – Presence of DNA

The assessment aims at answering the following question:

Is DNA detected or not detected in our product?

- ✓ Threshold of 10 ng of DNA/g  or mL of product
- ✓ Established based on the experience and previously assessed data
- ✓ Challenge? If the threshold cannot be reached (e.g. presence of nucleases)– explanations needed which will be evaluated on a case by case basis.

# 4. The Guidance document – Presence of DNA

## Most common flaws in the data provided?

- Missing data or samples not correct
- Missing the right controls or controls not done correctly
- Missing information in the reports/not clear that do not allow conclusions (lysis step, LOD, pictures bad quality)

# THANKS FOR YOUR ATTENTION