Determination of the antimicrobial activity of amino acid zootechnical mix

Three different batches examined

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Institute: Wageningen Food & Biobased Research - Food Technology

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Experiments were carried out by Nutricontrol

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Contents

Summary

Metex-Noovistago (MNG) would like to examine the antimicrobial activity of a new product using requirements from EFSA "Technical Guidance, Microbial Studies, Prepared by the Panel on Additives and Products or Substances used in Animal Feed", EFSA-Q-2008-461, adopted on 21 October 2008. To this aim three different samples of the Amino acid zootechnical mix were tested for their antimicrobial activity using the two-fold dilution method in broth following the recommendation broth in the referred EFSA Technical Guidance. For the antimicrobial activity tests, five different microorganisms were used to determine the antimicrobial activity of the mixture. The minimum inhibitory activity was determined by the third party - Nutricontrol.

The three batches of the amino acid zootechnical mix were shipped from WFBR location to the third party (Nutricontrol) and handled as follows to execute the experimental protocol determining their antimicrobial activity:
Broth micro-or macro-dilution is one of the most basic antimicrobial susceptibility testing methods. The procedure involves preparing two-fold dilutions of the antimicrobial agent in a liquid growth medium dispensed in a tube. The test material was incubated during 30 minutes at 90°C. After cooling, a pH correction and filtration, the analysis in aqueous BPW extract was carried out. Products were diluted in duplicate per bacteria culture and subsequently incubated under aerobic conditions at 37°C. The test tubes were visually judged after 24 hours and after 48 hours of incubation.
The highest dilution used was: 30 gram product + 300 ml aqueous solution (BPW was used), i.e. 9.1%. this was followed by two-fold dilutions.

Based on the above described experiments the following results were observed:
For the batches AA Mix#1 and AA Mix#2, after 24 hours of incubation at 37°C, there was no antimicrobial effect observed by any concentration tested, and no MIC value could be derived. For AA Mix#3 a MIC value of ≥ 9.1% was observed providing inhibition to the growth of *B. subtilis* ATCC 6633 in one of the duplicate tubes. This was not observed for the other cultures *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212.
After 48 hours of incubation at 37°C, no antimicrobial effect was detected and no MIC value for the samples of batch AA Mix#1, batch AA Mix#2 and batch AA Mix#3 could be derived.

1 Introduction

Metex-Noovistago intends to produce a new feed product and bring it to the market. Before the product can be put on the market, a study to perform the antimicrobial activity of the product needs to be in place. The antimicrobial activity study needs to be performed according to the Technical Guidance, Microbial Studies, Prepared by the Panel on Additives and Products or Substances used in Animal Feed, EFSA-Q-2008-461, adopted on 21 October 2008. In order to measure the antimicrobial activity, the Minimum Inhibitory Concentration (MIC) is being determined, which is the lowest concentration of the compound needed to inhibit microbial growth. The new product of Metex-Noovistago under study is a mixture of three amino acids (L-arginine (L-Arg), L-glutamine (L-Gln), L-threonine (L-Thr)) and dry plant extract (grape extract of Vitis Vinifera spp. Vinifera). In this report, the above described mixture will be referred as amino acid zootechnical mix.

The objective of this study was to determine the antimicrobial effect of the amino acid zootechnical mix. This was experimentally determined against 5 different microorganisms, being *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633 on three different batches of the amino acid zootechnical mix.

The experimental part on the determination of the antimicrobial activity was outsourced to a third party-Nutricontrol, while results and findings were analysed by WFBR to draw conclusions and assemble them to the present report.

2 Methods

The samples of the three different batches, namely AA Mix#1, AA Mix#2 and AA Mix#3, were taken at Research Diet Service on 19th October 2021, packed in plastic bags and stored in a dark, cooled room (temperature 5-7°C). On 22nd December 2021 the samples were shipped to Nutricontrol, a third party which carried out the determination of the antimicrobial activity.

Experimental approach according to the Technical Guidance EFSA-Q-2008-461 is provided next:
"For those substances whose antimicrobial effects are unknown, tests should be made to assess the inhibitory activity (minimum inhibitory concentration, MIC) against a list of reference strains known to be susceptible to clinically relevant antibiotics, ionophores or biocides (e.g., *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633). The MIC should be determined, according to standardised procedures, by using two-fold dilution procedures in agar or broth of the active antimicrobial substance. After incubation, the MIC is defined as the lowest concentration of the substance that inhibits microbial growth."

Third party (Nutricontrol) followed the above described recommendation and in the next paragraph we provide in short the key specifics. The detailed description, carried out by Nutricontrol for the execution of these tests is given in Annex 1.

Broth micro-or macro-dilution is one of the most basic antimicrobial susceptibility testing methods. The procedure involves preparing two-fold dilutions of the antimicrobial agent in a liquid growth medium dispensed in a tube. The test material was incubated during 30 minutes at 90°C. After cooling, a pH correction and filtration, the analysis in aqueous BPW extract was carried out. Products were diluted in duplicate per bacteria culture and subsequently incubated under aerobic conditions at 37°C. The test tubes were visually judged after 24 hours and after 48 hours of incubation.
The highest dilution used was: 30 gram product + 300 ml aqueous solution (BPW was used), i.e. 9.1%. this was followed by two-fold dilutions.

3 Results

The Minimal Inhibitory Concentration (MIC) results are given in table 1.

Table 1 Overview MIC results of three different batches amino acid zootechnical mix

	Batch AA Mix#1 (max 9.1%)	**Batch AA Mix#2 (max 9.1%)**	**Batch AA Mix#3 (max 9.1%)**
After 24 h incubation	No antimicrobial effect No MIC value	No antimicrobial effect No MIC value	The MIC value is ≥ 9.1% (w/v)[1]
After 48 h incubation	No antimicrobial effect No MIC value	No antimicrobial effect No MIC value	No antimicrobial effect No MIC value

[1)] The undiluted product inhibited only the growth of *B. subtilis* in one of the two tubes tested.

The blank and positive control showed the correct results.

Whenever the results of the turbidity was not clear, this was checked by plating the suspension on agar plates to check if the growth has indeed occurred.

For a more detailed description of the results, we refer to the report of Nutricontrol provided in Annex 1.

4 Conclusions

The antimicrobial activity of the Amino acid zootechnical mix was tested against 5 different microorganisms, being *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633.

After 24 hours of incubation at 37°C, there was no antimicrobial effect observed, and therefore no MIC value could be derived for the samples of the batch AA Mix#1 and AA Mix#2. For AA Mix#3 there was inhibition observed for the undiluted sample after 24 hours of incubation at 37°C, therefore this concentration of 9.1% was considered as a MIC value. The undiluted product (i.e. 9.1%) inhibited the growth of *B. subtilis* ATCC 6633 in one of the two tubes. This was not observed for the other species *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212.
No inhibition of microorganisms was observed at the lower tested dilutions.

Literature

Technical Guidance, Microbial Studies, prepared by the Panel on Additives or Substances used in Animal Feed, EFSA Journal (2008) 836, 1-3

Annex 1 Report Nutricontrol

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HR22.002

Project code: NC-671.01.V.07

MIC test on *E. coli*, *P. aeruginosa*, *S. aureus*, *E. faecalis* and *B. subtilis*

Veghel, January 14th 2022

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Project name	: MIC test on *E. coli*, *P. aeruginosa*, *S. aureus*, *E. faecalis* and *B. subtilis*.
Project number	: 671.01.V.07
Principal	: WUR, T. Verkleij
Account manager	: M. de Groot
Group	: Microbiologie/Biochemie
Projectleader	: S. Arts – van den Hoven
Projectteam	: S. Arts – van den Hoven, E. van Haaren
Date	: 14-01-2022
Version	: Version 1

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Date : 14-01-2022

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Agreed by reviewer NutriControl

Name: T. van der Vorst
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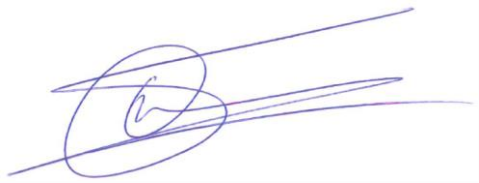

Agreed by commercial manager NutriControl

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1. Introduction

Wageningen Food & Biobased Research requested NutriControl to study the antimicrobial effect of three products according to “Technical Guidance, Microbial Studies, Prepared by the Panel on Additives and Products or Substances used in Animal Feed”, EFSA-Q-2008-461, adopted on 21 October 2008.

Description according to Technical Guidance EFSA-Q-2008-461:
“For those substances whose antimicrobial effects are unknown, tests should be made to assess the inhibitory activity (minimum inhibitory activity, MIC) against a list of reference strains known to be susceptible to clinically relevant antibiotics, ionophores or biocides (e.g., *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633). The MIC should be determined, according to standardized procedures, by using two-fold dilution procedures in agar or broth of the active antimicrobial substance. After incubation, the MIC is defined as the lowest concentration of the substance that inhibits microbial growth.”

2. Materials and methods

The following preparations have been tested and were registered as given below:

Received: 22-12-2021:

1. M22000066001: AA1
2. M22000066002: AA2
3. M22000066003: AA3

Product preparation according to standardized procedure:

- 30 gram product + 300 ml aqueous solution (BPW was used), i.e. 9.1%.
- 30 minutes incubation at 90°C
- Cooling
- pH correction of extracts to pH 7.0 ± 0.2
- Filtration
- Analysis in aqueous BPW extract

The products (extracts) were diluted – in duplicate, per bacteria culture - in BPW, as given in the following scheme:

	%	*E. coli* ATCC 25922		*P. aeruginosa* ATCC 27853		*S. aureus* ATCC 25923		*E. faecalis* ATCC 29212		*B. subtilis* ATCC 6633		No bacteria culture	
BPW	0												
Extract in BPW	9.1												
2 times diluted	4.5												
4 times diluted	2.3												
8 times diluted	1.1												
16 times diluted	0.6												
32 times diluted	0.3												

Fresh overnight cultures - of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212 and *B. subtilis* ATCC 6633 - were used to inoculate a BPW dilution at a start level as given below.

	log cfu/ml:
• *E. coli* ATCC 25922	5.41
• *P. aeruginosa* ATCC 27853	5.24
• *S. aureus* ATCC 25923	4.88
• *E. faecalis* ATCC 29212	5.24
• *B. subtilis* ATCC 6633	4.45

The tubes were incubated at 37°C, under aerobic conditions. The MIC test tubes were visually read after 24 hours incubation and after 48 hours incubation.

3. Results and discussion

pH correction to pH 7.0 ± 0.2:

1.	M22000066001, AA1	pH 8.80 to pH 7.02
2.	M22000066002, AA2	pH 8.82 to pH 7.04
3.	M22000066002, AA3	pH 8.74 to pH 7.03

All MIC (Minimal Inhibitory Concentration) results are given in appendix A.
All performed blank and positive controls showed the correct results.

Due to the dark colour of the extract and precipitate formation, it was difficult to visually determine turbidity of the medium in the undiluted extract (see "±" i.e. "uncertain turbidity" results in appendix A).
Also, because of precipitation of the product, it was difficult to visually detect growth of *S. aureus* (grows at the bottom of the tube due to the microaerophilic character of the microorganism) in the 2x and 4x diluted sample.
Therefore, in addition to visual assessment after 24 hours, a 10 µl inoculation from each tube was performed on blood agar (incubation 24 hours at 37°C) to observe growth.

<u>Product M22000066001, AA1:</u>
24 hours incubation:
No inhibition of microorganisms was observed.
48 hours incubation:
No inhibition of microorganisms was observed.

<u>Product M22000066002, AA2:</u>
24 hours incubation:
No inhibition of microorganisms was observed.
48 hours incubation:
No inhibition of microorganisms was observed.

Product M22000066003, AA3:

24 hours incubation:

The undiluted product (i.e. 9.1%) inhibits the growth of *B. subtilis* ATCC 6633 in one of the two tubes. This was not observed for the other cultures *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212.

No inhibition of microorganisms was observed at the other dilutions.

48 hours incubation:

No inhibition of micro-organisms was observed.

4. Conclusion

After 24 hours:

- M22000066001, AA1: there is no antimicrobial effect, and no MIC value
- M22000066002, AA2: there is no antimicrobial effect, and no MIC value
- M22000066003, AA3: the MIC value is ≥ 9.1%.

After 48 hours, there is no antimicrobial effect, and no MIC value.

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Appendix A. MIC results

Results expressed as turbidity after 24 h incubation and 48 h incubation (24h / 48h)

- = no turbidity
+ = turbidity
± = uncertain turbidity (only after 24 h)

	E. coli ATCC 25922		*P. aeruginosa* ATCC 27853		*S. aureus* ATCC 25923		*E. faecalis* ATCC 29212		*B. subtilis* ATCC 6633		No bacteria culture (blank controls)	
Tube	1	2	1	2	1	2	1	2	1	2	1	2
Positive controls												
BPW	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
M22000066001	**AA1**											
Extract in BPW	± / +	± / +	± / +	± / +	± / +	± / +	+ / +	+ / +	± / +	± / +	- / -	- / -
2 times diluted	+ / +	+ / +	+ / +	+ / +	± / +	± / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
4 times diluted	+ / +	+ / +	+ / +	+ / +	± / +	± / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
8 times diluted	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
16 times diluted	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
32 times diluted	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
M22000066002	**AA2**											
Extract in BPW	± / +	± / +	± / +	± / +	± / +	± / +	+ / +	+ / +	± / +	-[1] / +	- / -	- / -
2 times diluted	+ / +	+ / +	+ / +	+ / +	± / +	± / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
4 times diluted	+ / +	+ / +	+ / +	+ / +	± / +	± / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
8 times diluted	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
16 times diluted	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
32 times diluted	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
M22000066003	**AA3**											
Extract in BPW	± / +	± / +	± / +	± / +	± / +	± / +	+ / +	+ / +	± / +	± / +	- / -	- / -
2 times diluted	+ / +	+ / +	+ / +	+ / +	± / +	± / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
4 times diluted	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
8 times diluted	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
16 times diluted	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
32 times diluted	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -

[1] Uncertain result by visual reading was negative after confirmation on blood agar.
All other uncertain results were positive after confirmation on blood agar.

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The mission of Wageningen University and Research is "To explore the potential of nature to improve the quality of life". Under the banner Wageningen University & Research, Wageningen University and the specialised research institutes of the Wageningen Research Foundation have joined forces in contributing to finding solutions to important questions in the domain of healthy food and living environment. With its roughly 30 branches, 5,000 employees and 10,000 students, Wageningen University & Research is one of the leading organisations in its domain. The unique Wageningen approach lies in its integrated approach to issues and the collaboration between different disciplines.