Determination of the antimicrobial activity of the amino acid L-isoleucine

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Determination of the antimicrobial activity of the amino acid L-isoleucine

Three different batches examined

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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 one of their amino acids, L-isoleucine, using the 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 L-isoleucine were tested for their antimicrobial activity using the two-fold dilution method in broth, following the recommendation in the referred EFSA Technical Guidance. For the antimicrobial activity tests, five different microorganisms were used to determine the antimicrobial activity of the amino acid. After incubation the minimum inhibitory activity was determined. The determination of the antimicrobial activity was carried out by a third party - Nutricontrol.

The three batches of the amino acid L-isoleucine were shipped from WFBR location to the third party (Nutricontrol). The batch numbers were 210912, 210923 and 210924.
Broth micro-or macro-dilution is one of the most basic antimicrobial susceptibility testing methods. The procedure is described below to execute the experimental protocol determining their minimum inhibitory activity.
The procedure involves preparing two-fold dilutions of the antimicrobial agent in a liquid growth medium dispensed in a tube. The test material (diluted with aqueous solution 1/10) was incubated for 30 minutes at 90°C. After cooling, a pH correction and filtration, the analysis in the aqueous BPW (buffered peptone water) solution 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 concentration tested was 9.1% (start dilution was 30 gram product + 300 ml aqueous solution).

Based on the above described experiments, the following results were observed:
- After 24 hours of incubation at 37°C, no inhibition of micro-organisms was observed.
- After 48 hours of incubation at 37°C, no inhibition of micro-organisms was observed.

Conclusion: 9.1% or lower concentrations of the tested amino acid L-isoleucine did not show any antimicrobial activity in these experiments, which were designed according to the EFSA guideline 'Technical Guidance, Microbial Studies, Prepared by the Panel on Additives and Products or Substances used in Animal Feed'.

1 Introduction

Metex Noovistago (MNG) would like to examine the antimicrobial activity of one of their amino acids, L-isoleucine, using the 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 L-isoleucine were tested for their antimicrobial activity using the two-fold dilution method in broth following the recommendation in the referred EFSA Technical Guidance.

The objective of this study was to determine the antimicrobial effect of the amino acid L-isoleucine. 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 L-isoleucine.

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 L-isoleucine (batch 210912, 210923 and 210924) were received at Wageningen Food & Biobased Research, and stored in a dark, cooled room (temperature 5-7°C). On 06th July 2022 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."

The third party (Nutricontrol) followed the above described recommendation and in the next paragraph we provide in short the key specifics. The detailed description for the execution of these tests carried out by Nutricontrol 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 for 30 minutes at 90°C to dissolve the amino acid. 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 concentration used was: 30 gram product + 300 ml aqueous solution (BPW was used), i.e. 9.1%. this was followed by two-fold dilutions, the highest dilution was 32 times (i.e. 0.3%).

3 Results

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

Table 1 Overview MIC results of three different batches amino acid L-isoleucine

	Batch L-isoleucine 210912	**Batch L-isoleucine 210923**	**Batch L-isoleucine 210924**
After 24 h incubation	No antimicrobial effect	No antimicrobial effect	No antimicrobial effect
	No MIC value	No MIC value	No MIC value
After 48 h incubation	No antimicrobial effect	No antimicrobial effect	No antimicrobial effect
	No MIC value	No MIC value	No MIC value

The blank and positive control showed the correct results.

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 L-isoleucine 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 or 48 hours of incubation at 37°C, there was no antimicrobial effect observed, by concentrations tested (highest 9.1%), and therefore no MIC value could be derived for the samples of all three batches L-isoleucine (210912, 210923 and 210924.).

It can be concluded that at 9.1% or lower concentrations of the tested amino acid L-isoleucine not any antimicrobial activity was determined in these experiments, which were designed according to the EFSA guideline 'Technical Guidance, Microbial Studies, Prepared by the Panel on Additives and Products or Substances used in Animal Feed'..

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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HR22.031

Kenmerk: OFF-20220623-MDG-001020

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

Veghel, July 21st 2022

NutriControl
analytical solutions

Project name	: MIC test on *E. coli*, *P. aeruginosa*, *S. aureus*, *E. faecalis* and *B. subtilis*.
Kenmerk	: OFF-20220623-MDG-001020
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	: 21-07-2022
Version	: Version 1

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Name: S. Arts – van den Hoven
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Agreed by reviewer NutriControl

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Agreed by commercial manager NutriControl

Name: M. de Groot
Date : 21-07-2022

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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: 07-07-2022:

1. Isoleucine, B: 210912
2. Isoleucine, B: 210923
3. Isoleucine, B: 210924

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	5.33
• *P. aeruginosa* ATCC 27853	5.18
• *S. aureus* ATCC 25923	5.38
• *E. faecalis* ATCC 29212	5.22
• *B. subtilis* ATCC 6633	5.05

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.	Isoleucine, B: 210912	pH 6.82 to pH 6.96
2.	Isoleucine, B: 210923	pH 6.81 to pH 7.01
3.	Isoleucine, B: 210924	pH 6.85 to pH 6.98

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

Product Isoleucine, B: 210912:
24 hours incubation:
No inhibition of microorganisms was observed.
48 hours incubation:
No inhibition of microorganisms was observed.

Product Isoleucine, B: 210923:
24 hours incubation:
No inhibition of microorganisms was observed.
48 hours incubation:
No inhibition of microorganisms was observed.

Product Isoleucine, B: 210924:
24 hours incubation:
No inhibition of microorganisms was observed.
48 hours incubation:
No inhibition of microorganisms was observed.

4. Conclusion

After 24 and 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

	E. coli ATCC 25922		P. aeruginosa ATCC 27853		S. aureus ATCC 25923		E. faecalis ATCC 29212		B. subtilis ATCC 6633		No bacteria culture	
Controls												
BPW	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
Isoleucine, B: 210912												
Extract in BPW	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
2 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
4 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
8 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
16 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
32 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
Isoleucine, B: 210923												
Extract in BPW	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
2 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
4 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
8 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
16 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
32 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
Isoleucine, B: 210924												
Extract in BPW	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
2 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
4 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
8 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
16 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
32 times diluted	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-

To explore the potential of nature to improve the quality of life

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