NeoGiANT

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PROJECT INFORMATION

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List of participants:

No.	Acronym	Participant organisation name	Country
1 (Coord)	USC	Universidade de Santiago de Compostela	Spain
2	MRI	Moredun Research Institute	United Kingdom
3	IBPRS	Instytut Biotechnologii Przemysłu Rolno-Spożywczego im. prof. Wacława Dąbrowskiego	Poland
4	VRI	Veterinary Research Institute	Czech Republic
5	MATE	Nemzeti Agrárkutatási és Innovációs Központ	Hungary
6	FUB	Freie Universität Berlin	Germany
7	FCUP	Universidade do Porto – Faculdade de Ciências	Portugal
8	ULL	Universidad de La Laguna	Spain
9	UNE	Asociación Española de normalización	Spain
10	JU	Jihočeská Univerzita	Czech Republic
11	CONICET	Consejo Nacional de Investigaciones Científicas y Técnicas	Argentina
12	ASAJA	Asociación Agraria de Jóvenes Agricultores	Spain
13	ATM	Anitom S.L	Belgium
14	i-GRAPE	i-GRAPE	Spain
15	CTA	Contactica S.L	Spain
16	NUS	Nutrition Science	Belgium
17	CZV	CZ VACCINES	Spain
18	LBE	LIFEBIOENCAPSULATION SL	Spain
19	BIAN	BIANOR BIOTECH	Spain
20	MAGA	MAGAPOR S.L.	Spain

DELIVERABLE DETAILS

Document Number:	D3.1
Document Title:	MIC and IC50 for the tested microbial species using the polyphenolic extracts
Dissemination level	PU – Public
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WP:	WP3
Task:	Task 3.1
Author:	UNIVERSITY OF SANTIAGO DE COMPOSTELA
Abstract:	This document corresponds to the deliverable D3.1 MIC and IC50 for the tested microbial species using the polyphenolic extracts. It covers the results of the antimicrobial tests for several bacterial species using 4 different polyphenolic extracts: Ethyl lactate 50:50 water, Propylene glycol 50:50 water, Ethanol 50:50 water and Ethanol 50:50 water (ethanol removed)

Version	Date	Change
V1	27/06/2022	

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1. BACKGROUND

Antimicrobial tests *in vitro* have been performed using four polyphenolic extracts provided by WP2, with different polyphenolic contents depending on the extraction solvent:

Ethyl lactate 50:50 water (EL)

Propylene glycol 50:50 water (PG)

Ethanol 50:50 water (Et)

Ethanol 50:50 water (ethanol removed) (Etr)

2. BACTERIAL STRAINS AND METHODOLOGY

The microorganisms tested included gram-positive and gram-negative bacterial strains, being *Staphylococcus aureus* ATCC 25922 and *Escherichia coli* ATCC 25922 the reference strains to be used by all the groups involved in the antimicrobial tests, as a control of the inter-laboratory reproducibility.

Bacterial strains:

Staphylococcus aureus ATCC 25923

Escherichia coli ATCC 25922

Pseudomonas aeruginosa ATCC 27853

Staphylococcus hyicus DSM 20459

Bacillus subtilis ATCC 6633

Enterococcus faecalis ATCC 29212

Aeromonas salmonicida subsp. salmonicida CECT 894

Aeromonas hydrophila ATCC 7966

Proteus mirabilis ATCC 29906

Streptococcus suis ATCC 7007965

Citrobacter freundii ATCC 43864

Streptococcus uberis CECT 994

The antimicrobial tests were performed according to the EUCAST recommendations, using the Alamar-Blue colorimetric/fluorometric method, and reading the plates by fluorometry. The methodology involves the incubation of the cells in Müller-Hinton broth supplemented with different concentrations (ranging from 0,625 to 20%) of the antimicrobial substance to be evaluated and the appropriate amount of resazurin. This non-fluorescent molecule turns into the fluorescent molecule resorufin in the presence of metabolically active cells. This reaction allows quantifying the amount of living cells by measuring the fluorescence released by the resorufin produced during the process. This protocol offers values of Minimum Bactericidal Concentrations (MBC), so we calculate the Minimum Inhibitory Concentration (MIC) as the mean value between the MBC and the immediately previous concentration assayed. The values of IC50 have been obtained using the IC50 calculator (AAT Bioquest) program.

In order to distinguish between the antimicrobial effect of the polyphenolic content of the extracts and of the solvent itself, each extract has been tested in parallel with its solvent (vehicle). All tests were done in triplicate.

3. VALUES OF MIC AND IC50 FOR THE BACTERIAL SPECIES

Bacterial strain (Gram staining)	ETHYL LACTATE				PROPYLEN GLYCOL				ETHANOL				ETHANOLr			
	MIC (%)		IC50 (%)		MIC (%)		IC50 (%)		MIC (%)		IC50 (%)		MIC (%)		IC50 (%)	
	ext	ve	ext	ve	ext	ve	ext	ve	ext	ve	ext	ve	ext	ve	ext	ve
***S. aureus* (Gram +)**	0.9	7.5	1.03	3.4	3.75	15	2	3	≥20	≥20	1.07	0.96	1.8	n/a	1.5	n/a
***E. coli* (Gram -)**	12.5	7.5	7	4	20	15	20	9	≥20	≥20	15.72	4.93	≥20	n/a	18	n/a
***P. aeruginosa* (Gram-)**	3.75	1.88	0.7	0.9	≥20	15	1.9	7	-	-	-	-	≥20	n/a	n/a	n/a
***S. hyicus* (Gram +)**	0.9	7.5	0.4	4.7	1.88	≥10	1.4	n/a	-	-	-	-	0.9	n/a	0.6	n/a
***B. subtilis* (Gram +)**	1.1	3.75	0.9	3.8	1.88	≥10	1.8	n/a	-	-	-	-	0.8	n/a	0.7	n/a
***E. faecalis* (Gram +)**	1.9	1.9	1.9	1.4	3.8	≥10	3.8	n/a	-	-	-	-	3.8	n/a	3.7	n/a
***A. salmonicida* (Gram -)**	7.5	1.9	5	1.2	≥20	15	≥20	10	-	-	-	-	15	n/a	12.7	n/a
***A. hydrophila* (Gram -)**	7.5	7.5	2.92	0.69	≥20	≥20	12.64	19.75	≥20	≥20	12.82	n/a	-	n/a	-	n/a
***C. freundii* (Gram -)**	15	0.63	2.38	0.37	≥20	15	47.64	5.94	≥20	≥20	10.92	n/a	-	n/a	-	n/a
***P. mirabilis* (Gram -)**	7.5	0.63	1.04	0.35	15	15	1.75	9.09	≥20	≥20	8.87	28.83	-	n/a	-	n/a
***S. suis* (Gram +)**	3.75	0.625	1.59	0.83	7.5	15	2.16	8.71	7.5	≥20	4.66	1.55	-	n/a	-	n/a
***S. uberis* (Gram +)**	3.75	0.9	2.65	0.63	7.5	≥20	7.8	11.4	-	-	-	-	7.5	n/a	2.42	n/a

***ext** (extract); **ve** (vehicle)*
***n/a:** Both MICs and IC50 for the vehicle are not applicable in the case of Etr extract since the organic solvent has been removed. For the rest of the extracts tested, when the number of cells does not decline to 50% at the assayed concentrations, the values of IC50 are statistically not robust.*
-: tests have not been done

Ethyl lactate extract seems to be the one with the highest antimicrobial activity, since the values of MIC and IC50 are the lowest of all extracts assayed. Nevertheless, there are remarkable differences between the activity on Gram-positive and Gram-negative bacteria. With the only exception of the two species of *Streptococcus* assayed, the effect of the extracts on Gram-positive cells, are much better that those obtained with the vehicles, this suggesting that the antimicrobial effect is mostly due to the polyphenols present and not to the organic solvent in which the extract has been obtained. However, in the case of the Gram-negative species tested, the main antimicrobial effect seems to be due to the solvent, and certain cell protection provided by the polyphenols can be observed.

Similar results can be seen for the Ethanolic extract in which the solvent has been completely removed: it is very effective against the Gram-positive strains, but higher amounts of extract must be used to observe the antimicrobial effect on Gram-negative species. (Please, note that the volatilization of ethanol involves the concentration of the polyphenolic content by two-fold the one of the original ethanolic extract)

A similar tendency is observed for the Propylene glycol extract, although in this case, the extract seems to be effective against the *Streptococcus* species, whereas the vehicle has little or no effect.

The ethanolic extract exhibits the poorest values of MIC and IC50 for all the species tested, and so does the vehicle.

4. CONCLUSIONS

1. The methodology used has proved satisfactory reliability, since the results obtained by different research groups using the control species are the same
2. The extracts exhibit good inhibitory effect against the pathogenic bacteria assayed, being in general the Gram-positive strains more sensitive than the Gram-negative ones
3. Although the EL extract seems to exhibit the best activity against most strains, other extracts can be as effective depending on the bacterial species
4. The results of MIC and IC50 obtained show that the effect of the vehicles is important for certain species.
5. The antimicrobial effect of the extracts results as a combination of their polyphenolic content and the solvent in which it has been extracted, that acts as a co-ingredient.
6. When designing a formulation to prevent or treat a certain infectious disease, besides the animal's cells' tolerance to the solvent, the bacterial species involved in the infection must be considered in order to choose the most appropriate extract