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Molecular identification of lactic acid producing bacteria isolated from alheira, a traditional Portuguese fermented sausage †

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Abstract: Portuguese traditional fermented meat products constitute a valued economic and cultural heritage. The objective of this work was to screen the lactic acid bacteria (LAB) present in the alheira. 25 LABs were identified by Sanger sequencing of the 16S ribosomal gene. Sequencing results were aligned with sequences from the NCBI database using the BLAST algorithm. Genetic analysis showed a diverse lactic acid producing microbiome, and LABs from the family Lactobacillaceae and Leuconostocaceae were dominant, found in 64% of samples, while other organisms of the family Streptococcaceae and Enterococcaceae were found in 36% of samples. This work enabled the identification of LAB normally present in a traditional Portuguese product, as well as the desired technological characteristics that they can bestow to the product.

Keywords: microbial population diversity, food quality, food biotechnology, microbiome, fermented sausages

1. Introduction

Numerous types of fermented meat products exist in Europe, and they are highly appreciated by consumers. In addition to the economic importance of this supply chain, these products constitute a valued cultural heritage strongly linked to the identity of a population or to their production areas.

Alheira is a fermented meat sausage typical from the North region of Portugal and uses traditional technology for the manufacturing. Fermented sausages microbiome involves a complex interaction between LABs, which develops differently depending on the ripening process and raw materials used (Albano, H., et. al 2008).

Microbiomes involve an intrinsic and very sophisticated mechanism of bacterial interaction, which can create an environment either inhibitory for certain types of organisms, i.e., by production of metabolites, or promotion of bacteria that can survive in the same conditions and exchange benefits (Albano, H. et. al 2007, Taylor, B. C. et. al 2020).

Lactic acid bacteria (LAB) may vary across the different fermented products, resulting in a diversity of microorganisms that constitute the microbiome of the product, which are worth of characterization for improvement of quality control. Thus, the present study

aims to identify the LABs of alheira produced in the North region of Portugal using the Sanger sequencing technology and compare its molecular and physico-chemical properties.

2. Materials and Methods

LAB of fermented Portuguese Alheira sausages from the regions of Bragança, Mirandela, Vimioso, Mogadouro, Vinhais and Valpaços, were isolated (n=25) from 67 samples and stored at -80 ºC.

2.1. Reactivation of cryopreserved samples

Isolates were reactivated in 5 mL of Man, Rogosa and Sharpe (MRS) Broth and incubated at 37 ºC for 24 h. After incubation, 1.5mL of culture was transferred to eppendorfs and centrifuged at 10000 x g for 2 min; the process was repeated two times for each culture. The supernatant was discarded, and the pellet was kept at 4 ºC.

2.2. DNA extraction

Genomic DNA (gDNA) of samples was extracted using GF-1 Bacterial DNA Extraction Kit (Vivantis), with the optional RNA removal step. The DNA concentration and purity were analysed by 260/280 ratio.

2.3. 16S rRNA amplification

The primers used for amplification of the 16S rRNA gene were 27f 5'- AGA GTT TGA TCC TGG CTC AG -3' and 1492r 5'-CTA CGG CTA CCT TGT TAC GA-3'. The PCR cycle was 94 ºC for 2 min, followed by 30 cycles of 94 ºC for 10 sec, 55 ºC for 20 sec and 72 ºC for 1 min, using DFS-Taq DNA polymerase.

PCR products were visualized by electrophoresis on a 1 % (w/v) agarose gel, stained with ethidium bromide, purified with the GF-1 PCR Clean-up Kit (Vivantis) and used as template in the sequencing reactions. Quality of amplicon was measured by 260/280 ratio.

2.4. Sanger sequencing

Sequencing reactions used BigDyeTM Terminator v3.1 while purification of samples used SAM/BigDyeXTerminatorTM bead solution (ThermoFisher Scientific, Portugal). Capillary electrophoresis was run in SeqStudio Genetic Analyzer (Applied Biosystems, Portugal).

2.4. Sequence analysis

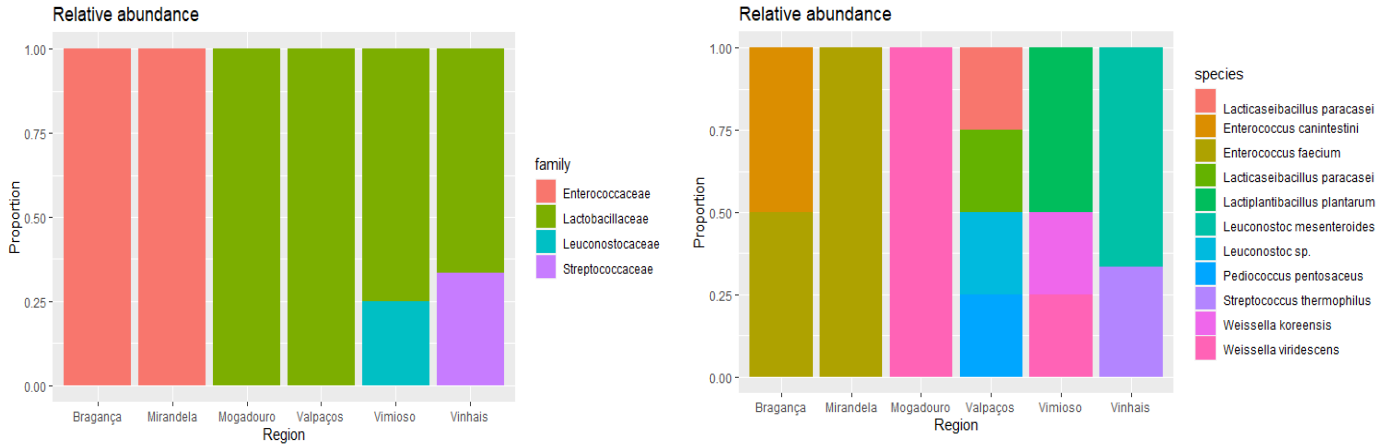
Sequence results were aligned with sequences from the NCBI database using the BLAST algorithm. Finally, sequences with identity higher than 85% were accepted as the best match for the LAB isolate.

2.4. Data analysis

Graphs were plotted using ggplot2, dplyr and AER packages using the software R (version 4.1.3).

3. Results

According to BLAST results from 16S rRNA sequencing of 25 samples, LABs from the family *Lactobacillaceae* and *Leuconostocaceae* were dominant in the microbiome of Alheiras, found in 64% of samples, while other organisms of the family *Streptococcaceae* and *Enterococcaceae* were found in 36% of samples (Figure 1). At the species level, *Enterococcus faecium* was the most abundant organism (28%), followed by *Lacticaseibacillus paracasei* (16%) and *Weissella viridescens* (12%).



(**a**) (**b**)

abundance of lactic acid bacteria isolated from alheira sausages per region at (a) family level and (b) species level.

Previous studies from our group have tested physicochemical properties of 17 out of 25 LABs used in this work (Faria, A.S. et al 2021), namely, proteolytic activity (PeoteolyticAct), acidifying capacity (pH6) and antimicrobial capacity according to different growth media (MRS or M17).

Results presented in Table 1 show that *Lactobacillus plantarum* strain MLG5-1 has the highest growth inhibition of *Staphylococcus aureus subsp. aureus* strain ATCC 6538, *Listeria monocytogenes* ATCC 35152 and *Salmonella enterica subsp. enterica serovar Typhimurium* strain ATCC 43971. Additionally, *Pediococcus pentosaceus* strain 56.5 had the highest L-lactic acid production (0.663 g/L). Additional LABs identified belong to the family *Lactobacillaceae* and *Enterococcaceae*.

Table 1. Physicochemical properties and growth media of lactic acid bacteria isolated from Portuguese alheira sausage, along with the corresponding identified strain. Variables: Description (retrieved sequence from the NCBI database), Species (scientific name of organism found), Agar media (selection media either MRS or M17), InhDiam_10ºC_Salmo (Diameter of Salmonella enterica tested at 10ºC), InhDiam_10ºC_Listeria (Diameter of Listeria monocytogenes tested at 10ºC), InhDiam_10ºC_Staphy (Diameter of Staphylococcus aureus tested at 10ºC), ProteolyticAct (Proteolytic activity in mm), pH (acidity of broth after 6 h), [LAC] (L-lactic acid concentration in g/L), methods for obtaining these values are described in Faria, A.S. et al 2021.

			Inhibition Diameter			Proteolytic Action	pH	[LAC]
Description	**Species**	**Agar Media**	**Salmonella**	**Listeria**	**Staphylococcus**			
Lactobacillus plantarum strain MLG5-1	Lactiplantibacillus plantarum	MRS	12.65	25.86	13.88	3.34	6.466	0.092
Lactobacillus plantarum strain 8277	Lactiplantibacillus plantarum	MRS	9,31	21,64	11,23	4,47	6,324	0,493
Lactobacillus paracasei subsp. paracasei strain MA34	Lacticaseibacillus paracasei subsp. paracasei	MRS	9,62	19,66	8,14	4,29	6,498	0,611
Lactobacillus paracasei M15-104	Lacticaseibacillus paracasei	MRS	9,98	15,65	10,78	0,00	6,496	0,610
Leuconostoc sp. THK-W39	Leuconostoc sp.	MRS	10,70	17,85	9,51	3,14	6,337	0,032
Leuconostoc sp. strain E1LBL6mb	Leuconostoc sp.	MRS	11,37	15,09	11,08	4,37	6,345	0,032
Pediococcus pentosaceus strain 56.5	Pediococcus pentosaceus	MRS	10,42	18,05	7,62	4,74	6,406	0,663
Pediococcus pentosaceus strain KS12	Pediococcus pentosaceus	MRS	10,87	20,72	10,76	5,48	6,388	0,029
Lactobacillus paracasei strain 8381	Lacticaseibacillus paracasei	MRS	10,38	17,38	10,25	4,81	6,412	0,646
Lacticaseibacillus paracasei strain Y526	Lacticaseibacillus paracasei	MRS	9,11	18,54	10,46	0,00	5,837	0,056
Leuconostoc mesenteroides strain 4486	Leuconostoc mesenteroides	MRS	10,23	21,19	10,90	1,30	6,399	0,019
Streptococcus thermophilus strain Y15	Streptococcus thermophilus	MRS	11,53	19,42	10,21	8,26	5,916	0,031
Enterococcus canintestini strain 735	Enterococcus canintestini	MRS	10,72	17,12	9,10	2,20	5,736	0,361
Enterococcus faecium strain KB-15	Enterococcus faecium	M17	4,35	11,66	5,24	1,11	5,564	0,270
Enterococcus faecium strain BL4-13	Enterococcus faecium	M17	3,43	11,79	4,49	0,00	5,591	0,499
Enterococcus faecium strain KB-15	Enterococcus faecium	M17	5,13	11,25	5,20	0,00	5,525	0,201
Enterococcus faecium strain gp8	Enterococcus faecium	M17	6,13	7,55	5,56	0,00	5,618	0,282

4. Discussion and Conclusion

The results obtained were expected given that previous studies report finding typical LAB, such as Weissella viridescens, Leuconostoc mesenteroides, Lacticaseibacillus paracasei, Pediococcus pentosaceus as the predominant organisms in Alheira, as well as organisms of the family Enterococcaceae, such as Enterococcus faecium probiotic strains (Albano, H., et. al 2008; Maria, Creciana et. al 2021). LAB acidity activity contributes to the physical stability of the food product and promotes protection against pathogens, creating a stable microbiological environment. Pediococcus pentosaceus and Lactobacillus plantarum strains appears as the highest L-lactic acid producers and with the highest antimicrobial activity. Genetic analysis of 25 samples showed a diverse lactic acid producing microbioma, which is variable for the different regions screened. The variability could be influenced by geographic region, manufacturing process, ripening process and raw materials used.

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Conflicts of Interest: The authors declare no conflict of interest.

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