biology and life sciences forum

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Proceeding Paper

Molecular Identification of Lactic Acid Producing Bacteria Isolated from Alheira, a Traditional Portuguese Fermented Sausage †

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† Presented at the 3rd International Electronic Conference on Foods: Food, Microbiome, and Health—A Celebration of the 10th Anniversary of Foods' Impact on Our Wellbeing, 1–15 October 2022; Available online: https://sciforum.net/event/Foods2022.

Abstract: Traditional Portuguese fermented meat products represent a valued economic and cultural heritage. The objective of this work was to screen the lactic acid bacteria (LAB) present in alheira sausages. Twenty-five LAB were identified by means of 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 LAB from the family *Lactobacillaceae* and *Leuconostocaceae* were dominant, found in 64% of samples, whereas other organisms of the family *Streptococcaceae* and *Enterococcaceae* were found in 36% of samples. This work enabled the identification of the LAB that are normally present in alheira, as well as the understanding of their association with the technological characteristics of this traditional fermented sausage.

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

Citation: Fernandes, N.; Faria, A.S.; Carvalho, L.; Choupina, A.; Rodrigues, C.; Cadavez, V.; Gonzales-Barron, U. Molecular Identification of Lactic Acid Producing Bacteria Isolated from Alheira, a Traditional Portuguese Fermented Sausage. *Biol. Life Sci. Forum* **2022**, *18*, 73. https://doi.org/10.3390/Foods2022-13035

Academic Editor: Antonello Santini

Published: 14 October 2022

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 represent a valued cultural heritage that is strongly linked to the identity of a population and to their production areas [1].

Alheira is a fermented meat sausage that is typical to the northern region of Portugal, and traditional technology is used for its manufacturing. The fermented sausages' microbiome involves complex interaction between LAB, which develops differently depending on the ripening process and raw materials used [2].

Microbiomes involve an intrinsic and very sophisticated mechanism of bacterial interaction, which can create an environment that is either inhibitory for certain types of organisms, i.e., via the production of metabolites, or which can promote bacterial growth and the exchange of mutual benefits [3]. Lactic acid bacteria (LAB) may vary among different fermented products, resulting in the diversity of the microorganisms that constitute the microbiome of the product, which are worthy of characterization in regard to the improvement of safety and quality control [4].

Thus, in the present study we aimed (1) to identify the LAB of alheira produced in the northern region of Portugal and to assess their technological properties, and (2) to study the antimicrobial capacity of LAB against *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Staphylococcus aureus*.

2. Materials and Methods

LAB from fermented Portuguese alheira sausages, sampled in the regions of Bragança, Mirandela, Vimioso, Mogadouro, Vinhais, and Valpaços, were isolated (n = 25) from 67 samples and stored at −80 °C.

2.1. Reactivation of Cryopreserved Samples

The LAB isolates were reactivated in 5 mL of Man, Rogosa, and Sharpe (MRS) broth (Himedia, Einhausen, Germany) and incubated at 37 °C for 24 h. After incubation, 1.5 mL of culture was transferred to Eppendorf tubes and centrifuged at 10,000× *g* for 2 min; the process was repeated two times for each culture. The supernatant was discarded, and the pellet was kept at 4 °C.

2.2. DNA Extraction

Genomic DNA (gDNA) was extracted from samples using a GF-1 Bacterial DNA Extraction Kit (Vivantis, Shah Alam, Malaysia), with the optional RNA removal step. The DNA concentration and purity were analyzed using the 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′ [5]. The PCR cycle was 94 °C for 2 min, followed by 30 cycles of 94 °C for 10 s, 55 °C for 20 s and 72 °C for 1 min, using DFS-Taq DNA polymerase (Bioron Life Sciences, Römerberg, Germany).

PCR products were visualized via electrophoresis on 1% (*w*/*v*) agarose gel, stained with ethidium bromide, purified with the GF-1 PCR Clean-up Kit (Vivantis, Shah Alam, Malaysia), and used as template in the sequencing reactions. The quality of amplicons was measured using the 260/280 ratio.

2.4. Sanger Sequencing

For sequencing reactions, we used a BigDyeTM Terminator v3.1 system, whereas for the purification of samples, we used a SAM/BigDyeXTerminatorTM bead solution (ThermoFisher Scientific, Oeiras, Portugal). Capillary electrophoresis was carried out using a SeqStudio Genetic Analyzer (Applied Biosystems, Porto, Portugal).

2.5. 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 matches for the LAB isolates.

2.6. Screening of LAB for Acidifying Capacity, Proteolytic Activity, and Antimicrobial Capacity

Previous studies [6] have tested the physicochemical properties of 17 out of the 25 LAB used in this work, namely, in-vitro proteolytic activity (in mm); L-lactic acid concentration (g/L); acidifying capacity (pH after 6 h); and antimicrobial capacity against *Salmonella enterica subsp. enterica serovar* Typhimurium strain ATCC 43971, *Listeria monocytogenes* ATCC 35152, and *Staphylococcus aureus subsp. aureus* strain ATCC 6538 at 10 °C, measured as inhibition diameter (mm) on MRS or M17 agar (Liofilchem, Abruzzi, Italy). The use of MRS or M17 agar to quantify in vitro susceptibility depended on the media on which the LAB isolate was recovered from the alheira sausage homogenate.

2.7. Data Analysis

A local alignment search was conducted with the BLAST tool to measure the similarity between sequences [7]. The prevalences of LAB were calculated with R software version 4.1.3 [8] and graphs were plotted using ggplot2 [9].

3. Results and Discussion

Figure 1 shows the relative abundance of lactic acid bacteria isolated from sausages by region (a) at the family level and (b) at the species level. According to the BLAST results from the 16S rRNA sequencing of 25 samples, LAB from the family *Lactobacillaceae* and *Leuconostocaceae* were dominant in the microbiome of alheira sausages, found in 64% of samples, whereas other organisms of the family *Streptococcaceae* and *Enterococcaceae* were found in 36% of samples (Figure 1a). At the species level (Figure 1b), *Enterococcus faecium* was the most abundant organism (28%), followed by *Lacticaseibacillus paracasei* (16%) and *Weissella viridescens* (12%).

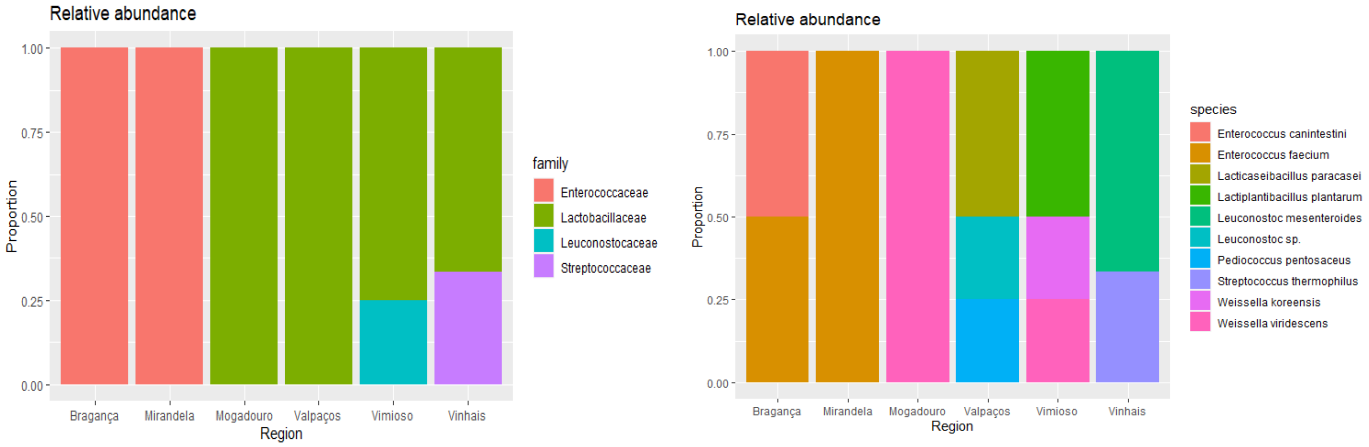

Figure 1. Relative abundance of lactic acid bacteria isolated from alheira sausages per region at (**a**) the family level and (**b**) the species level.

Table 1 shows the physicochemical properties and growth media of lactic acid bacteria isolated from alheira sausages, along with the corresponding identified species. The results showed that *Leuconostoc mesenteroides* had the highest proteolytic activity, followed by *Pediococcus pentosaceus* and *Lacticaseibacillus paracasei* (Table 1). Similar findings were reported by [10], who identified *Pediococcus pentosaceus* as having one of the highest proteolytic activitues among other LAB isolated from dry-cured ham.

At the species level, *Enterococcus faecium* presented a higher overall acidifying capacity, being able to decrease the pH to 5525 after 6 h in skim milk when compared to the other LAB, a result which may be influenced by L-lactic acid production (0.5 g/L). *Leuconostoc sp.* showed the lowest concentration of the lactic acid L-isomer (0.032 g/L); this result was expected since this LAB is a producer of the D-lactic acid isomer [11]

Lactobacillus plantarum had the highest antimicrobial capacity against the three pathogens tested, indicating that this LAB isolate could potentially be from a bacteriocinogenic strain [12]. On average, LAB isolated from alheira exhibited the highest inhibitory activity against *Listeria monocytogenes. Leuconostoc mesenteroides, Leuconostoc sp.*, and *Lacticaseibacillus paracasei* stood out as being the LAB species that appeared to have the highest inhibition potential against *Staphylococcus aureus* and *Salmonella enterica.*

Table 1. Physicochemical properties and growth media of lactic acid bacteria isolated from Portuguese alheira sausage, along with the corresponding identified species.

Species	Agar Media	Inhibition Diameter (mm)			Proteolytic Action (mm)	pH	Lactic Acid Concentration (g/L)
		Salmonella Typhimurium	*Listeria monocytogenes*	*Staphylococcus aureus*			
Lactiplantibacillus plantarum	MRS	12.65	25.86	13.88	3.34	6.466	0.092
Lactiplantibacillus plantarum	MRS	9.31	21.64	11.23	4.47	6.324	0.493
Lacticaseibacillus paracasei subsp. *paracasei*	MRS	9.62	19.66	8.14	4.29	6.498	0.611
Lacticaseibacillus paracasei	MRS	9.98	15.65	10.78	0.00	6.496	0.610
Leuconostoc sp.	MRS	10.70	17.85	9.51	3.14	6.337	0.032
Leuconostoc sp.	MRS	11.37	15.09	11.08	4.37	6.345	0.032
Pediococcus pentosaceus	MRS	10.42	18.05	7.62	4.74	6.406	0.663
Pediococcus pentosaceus	MRS	10.87	20.72	10.76	5.48	6.388	0.029
Lacticaseibacillus paracasei	MRS	10.38	17.38	10.25	4.81	6.412	0.646
Lacticaseibacillus paracasei	MRS	9.11	18.54	10.46	0.00	5.837	0.056
Leuconostoc mesenteroides	MRS	10.23	21.19	10.90	1.30	6.399	0.019
Leuconostoc mesenteroides	MRS	11.53	19.42	10.21	8.26	5.916	0.031
Enterococcus canintestini	MRS	10.72	17.12	9.10	2.20	5.736	0.361
Enterococcus faecium	M17	4.35	11.66	5.24	1.11	5.564	0.270
Enterococcus faecium	M17	3.43	11.79	4.49	0.00	5.591	0.499
Enterococcus faecium	M17	5.13	11.25	5.20	0.00	5.525	0.201
Enterococcus faecium	M17	6.13	7.55	5.56	0.00	5.618	0.282

4. Conclusions

Weissella viridescens, *Leuconostoc mesenteroides*, *Lacticaseibacillus paracasei*, and *Pediococcus pentosaceus* were the predominant organisms in alheira sausage, and organisms from the family *Enterococcaceae*, such as *Enterococcus faecium*, were more recurrent in the locations of Bragança and Mirandela.

Alheira acidification by LAB contributes to the physicochemical stability of the product and promotes protection against pathogens, creating a stable microbiological environment. *Enterococcus faecium* presented a higher overall acidifying capacity for the decrease in pH, and these bacteria, along with *Pediococcus pentosaceus*, were the highest L-lactic acid producers.

Listeria monocytogenes was the pathogen that most of the assayed LAB were capable of inhibiting. *Lactobacillus plantarum* had the highest antimicrobial capacity against the three pathogens tested. *Leuconostoc mesenteroides* had the highest proteolytic activity, followed by *Pediococcus pentosaceus* and *Lacticaseibacillus paracasei*.

Finally, genetic analysis of 25 samples showed a diverse lactic-acid-producing microbiome, which was variable among the different regions screened. This variability could be influenced by different geographic regions [13], manufacturing processes, ripening processes, and raw materials used.

Author Contributions: Conceptualization, N.F., V.C. and U.G.-B.; methodology, N.F., L.C., A.C., C.R. and U.G.-B.; software, N.F., L.C., V.C. and U.G.-B.; validation, C.R., V.C. and U.G.-B.; formal analysis, N.F., A.S.F., L.C., A.C., C.R. and U.G.-B.; investigation, N.F., A.S.F., L.C., A.C., C.R. and U.G.-B.; resources, A.C., C.R., V.C. and U.G.-B.; data curation, N.F., A.S.F., V.C. and U.G.-B.; writing—original draft preparation, N.F.; writing—review and editing, N.F., V.C. and U.G.-B.; visualization, V.C. and U.G.-B.; supervision, C.R., V.C. and U.G.-B.; project administration, V.C. and U.G.-B.; funding acquisition, V.C. and U.G.-B. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support through national funds FCT/MCTES (PIDDAC) to CIMO (UIDB/00690/2020 and UIDP/00690/2020) and SusTEC (LA/P/0007/2021). The authors are also grateful to the EU PRIMA program, the Portuguese Foundation for Science and Technology (FCT) for funding the ArtiSaneFood project (PRIMA/0001/2018).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Summary data available upon request.

Acknowledgments: U. Gonzales-Barron would like to thank the national funding by FCT, through the institutional scientific employment program-contract.

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

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