



An assessment of probiotic properties of *Lactobacillus plantarum* subsp. *plantarum* and *Lb. plantarum* subsp. *argenterotensis*

Guidone, A.¹, Zotta, T.¹, Ricciardi, A.¹, Ross, R. P.^{3,4}, Stanton, C.^{3,4}, Rea, M. C.^{3,4}, Parente, E.^{1,2}

¹ Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Università degli Studi della Basilicata, Potenza, Italy

² Istituto di Scienze dell'Alimentazione, Consiglio Nazionale delle Ricerche, Avellino, Italy

³ Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

⁴ Alimentary Pharmabiotics Centre, University College Cork, Ireland



INTRODUCTION

Probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2006). *Lactobacillus plantarum* is an industrially important lactic acid bacterium which is presently available in the market in probiotic products, functional and therapeutic foods and potential live oral vaccines. *Lb. plantarum* strains are able to survive during gastric transit and colonize the intestinal tract of humans and other mammals. *Lb. plantarum* strains are able to reduce the incidence of diarrhea, the inflammation, pain and constipation of irritable bowel syndrome, and might exert positive effect on immunity in HIV⁺ children and on the reduction of serum cholesterol and triglyceride levels. According to the guidelines reported in FAO/WHO (2006), *in vitro* tests to screen potential probiotics traits should evaluate the resistance to gastric acidity and to bile salts, the adherence to mucus and/or human epithelial cells and cell lines, the antimicrobial activity against potentially pathogenic bacteria and bile salt hydrolase activity. **The aim of this work was** to carry out a multivariate screening study for probiotic properties in 32 *Lactobacillus plantarum* strains previously isolated from different sources in order to identify strains with probiotic potential.

RESULTS

Probiotic features

Species	Strain	Source	A	B	C	D	E	F	G	H	I	bsh	msa
<i>Lplp</i>	895	SD										1	0
	947	SD										0	0
	1069	SD										1	1
	1505	SD										1	1
	38AA	FV										0	1
	C17	CH										1	1*
	DCU101	SI										1	1
	DPC1115	UN										1	1
	DPC1121	UN										0	1
	DPC1122	CH										0	1
	DPC2120	CH										1	0
	DPC2127	CH										0	1
	DPC2159	CH										0	1*
	DPC2183	CH										1	1*
	DPC2190	CH										1	0
	DPC4229	CH										0	1
	DPC6421	AN										1	0
	DPC6429	HU										1	1
	DPC6430	WM										1	1
	FSM170	CH										0	1*
<i>Lpla</i>	MTD2S	SD										1	1
	MTFIL	SD										1	0
	NCFB340	SI										1	1
	P1.5	OL										1	1*
	S12	CH										1	1
	S85	VE										1	0
	UBS3	WI										1	1
	UT2.1	WI										1	0
	WFCS1	HU										1	1
	DK36	FV										1	0
DKO22 [†]	FV										0	0	
NCIMB12120	FV										0	0	

Strains and isolation sources: AN, animal isolate; CH, cheese; FV, fermented vegetables; HU, human isolate; OL, olives; SD, sourdough; SI, silage; UN, unknown; VE, vegetables; WI, wine; WM, water milking. Probiotic activities: none, white cell; weak, light gray cell; intermediate, gray cell; intense, black cell. A, BSH activity; B, intensity of yeast agglutination without methyl- α -D-mannopyranoside; C, intensity of yeast agglutination in the presence of methyl- α -D-mannopyranoside; D, bile resistance; E, lysozyme resistance; F, β -galactosidase activity; G, Surface hydrophobicity; H, biofilm; I, resistance to SG. *bsh* and *msa* genes: 0, 1, 1* indicate the absence, presence and presence with specific bands of expected amplicons.

The intensity of probiotic features varied significantly among strains and was lower in *Lb. plantarum* subsp. *argenterotensis*. Non hierarchical cluster analysis was used to find reasonable breakpoints (shown as different shading levels in the figure above) in the distribution of continuous properties (lysozyme, gastric juice and bile salts resistance, surface hydrophobicity, ability to form biofilm, β -galactosidase activity) while a nominal scale was used for the others. The highest value was always assigned to groups with most desirable properties and the scores for individual variables were summed to obtain a global probiotic score.

CONCLUSIONS

This study showed a large heterogeneity in probiotic properties confirming that they are strain related. Multivariate statistical methods allowed us to obtain a more comprehensive evaluation of the pattern of potential probiotic features, compared to simple univariate tests on single features. Strains with promising patterns of probiotic properties can be easily isolated from different food sources but *in vitro* and *in vivo* studies of critical abilities (adherence to cell lines and colon) and of health related effects are needed.

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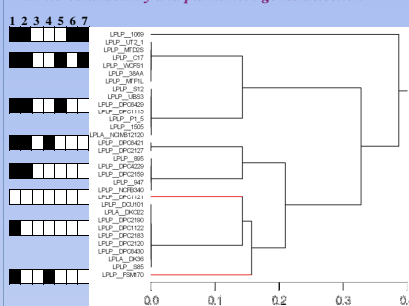
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MATERIALS AND METHODS

Strains: 29 *Lb. plantarum* subsp. *plantarum* and 3 *Lb. plantarum* subsp. *argenterotensis* strains were previously identified by Multiplex-PCR and PFGE. **Analysis of genes supporting probiotic action:** The presence of *bsh* and *msa* genes encoding for the bile salt hydrolase (BSH) and for the mannose-specific adhesion (MSA) and of 7 genes (*orf345*, *plnA*, *plnC8a/b*, *plnC8f*, *plnEF*, *plnJK*, *plnN*) of the *pln* locus were evaluated. **In-vitro tests:** Resistance to biological barriers such as simulated saliva (electrolyte solution containing 100 mg/L of lysozyme), simulated gastric juice (0.3% w/v pepsin, 0.5% w/v NaCl, pH 2) and bile salts (MRS broth with 0.2, 0.5 or 1% w/v of bile salts) were evaluated. Probiotic characteristics as deconjugation of sodium taurodeoxycholate hydrate (TDCA) bile salts, surface hydrophobicity, capability to form biofilm, the ability to use lactose (Bromocresol Purple Lactose Agar), β -galactosidase activity, agglutination of yeast cells, EPS production, inhibitory activity (against *L. innocua* B186/26, *Lb. pentosus* STP, *Lb. plantarum* DPC1121 and the cross inhibition within the 32 *Lb. plantarum* strains using a deferred antagonism assay and a well-diffusion agar assay respectively) and antibiotic resistance (to gentamicin, tetracycline, erythromycin and chloramphenicol) were also tested. **Statistical analysis** was carried out using Systat 13 (Systat Inc., Chicago, IL). For each probiotic feature a semiquantitative score (1-3; 1 weak - 3 strong) was calculated using k-means clustering on the Euclidean distance matrix to identify cut-points for clusters. The matrix of semiquantitative scores was used for multidimensional scaling using the Spearman rank order correlation coefficient.

Antimicrobial activity and *pln* genes detection



Cluster analysis on *plnJK* (1), *plnEF* (2), *plnC8a/b* (3), *orf345* (4), *plnA* (5), *plnC8f* (6), *plnN* (7) genes/operons encoding for the plantaricins JK, EF, NC8, IS1, for the pheromones: A, NC8F and for the peptide PlnN revealed 8 genetic groups (plantaritypes). Each plantaritype contained respectively 6, 7, 5, 3 and 9 strains with the same combination of plantaricins (from none to 4, in different combinations, shown as a black and white pattern on the left).

❖ Plantaricins and plantaricin-like pheromones play an important role in *Lb. plantarum* ecology. Several plantaricins are potent inhibitors of pathogenic microorganisms, while others play a role in quorum-sensing. As for many other bacteriocins, plantaricin production is controlled by auto-induction, and it is considered a probiotic trait (Dobson et al., 2012).

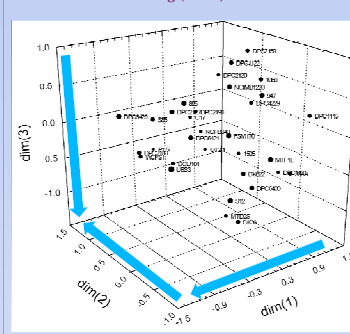
❖ None of the *Lb. plantarum* strains used in this study was capable of inhibiting the indicator strains.

❖ Plantaritypes were not related to isolation source nor to grouping of strains by PFGE (not shown).

❖ *Lb. plantarum* subsp. *plantarum* 1069 and FSM170 had unique combinations of genes, while DPC1121 had none of the plantaricins genes tested in this study.

❖ We confirmed the remarkable plasticity and the presence of different types of *pln* loci among our 32 strains isolated from different sources without finding any correlation with the origin of the strains.

Multidimensional scaling (MDS)



❖ Heterogeneity in probiotic properties among the different strains of *Lb. plantarum* was revealed.

❖ To obtain a graphical representation of the results and to evaluate the relationship between factors affecting probiotic properties, a MDS was carried out on the Pearson's r correlation matrix:

- Bile tolerance and yeast agglutination significantly affected the distribution of strains in the first dimension, surface hydrophobicity, biofilm and tolerance to gastric juice affected the second dimension while in the third dimension the principal factor affecting the distribution was the bile resistance. As a consequence, strains with the best combination of probiotic properties were located towards the left and the back of the plot.
- MDS analysis helped us to evaluate the relationship between factors affecting probiotic properties and to discover the strains with the best probiotic properties.
- A group of 9 strains (C17, DCU101, DPC2127, DPC2183, DPC2190, DPC6429, P1.5, S85 and WFCS1) isolated from different sources had the highest probiotic scores (17-19).

Antibiotic resistance

Probiotics must be safe for human consumption. According to the guidelines reported in EFSA (2008), *Lb. plantarum* strains can be categorised as susceptible or resistant to antimicrobials when they are respectively inhibited or not at breakpoint level of a specific antimicrobial. For *Lb. plantarum* strains the breakpoints values for erythromycin, chloramphenicol, gentamicin and tetracycline are respectively: 1, 8, 16 and 32 mg/L. We found that only strains C17 and S85 strains were both resistant to tetracycline and only C17 was also resistant to erythromycin. Genotypic characterization of antibiotic resistance determinants is in progress.

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