

***In vitro* Fermentation and Bifidogenic Potential of Galacturonic Acid**

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The first aim of our study was to screen some human lactic acid bacteria on the basis of probiotic characteristics. The second aim was therefore to study the bifidogenic properties of D-galacturonic acid, major component of pectin via their *in vitro* fermentation by four human bacteria strains; *Listeria monocytogenes*, *Staphylococcus aureus* as potential gut pathogenic bacteria and *Lactobacillus reuteri*, *Streptococcus thermophilus* as potential probiotic gut bacteria and assessing the ability of the candidate probiotic influenced the auto-aggregation of probiotic candidates, in second time the eventual effect of D-galacturonic acid on EPS production of the two probiotic candidate on various carbon source. Our results show that characterized lactic strains possess interesting probiotic properties. Indeed, they revealed a good thermoresistance (65°C) and they were both tolerant to the acidity (pH 2.5). Our study revealed during this *in vitro* study that galacturonic acid possess remarkable probiotic characteristic. In fact, it both exerted a selective stimulation on the beneficial strains 9.58 Log FCU/ml and 8.63 FCU/ml respectively for *Lactobacillus reuteri*, *Streptococcus thermophilus* and inhibited from other part the growth of the pathogenic ones. The obtained results revealed that galacturonic acid increased significantly the probiotic EPS production and it was estimated for a long phase growth.

Keywords: Galacturonic acid, Lactic acid bacteria, Probiotic, Prebiotic, Exopolysaccharids.

The term « pectin » refers to a group of complex polysaccharides that are components of the cell walls of most higher plants¹. Their main structural features include a backbone of (1 → 4)-linked -d-galacturonic acid units. These “smooth” homogalacturonic regions are interrupted by « hairy » rhamnogalacturonic regions in which galacturonic acid residues are interspersed with (1 → 2)-linked-1-rhamnopyranosyl residues. Some rhamnosyl residues are substituted by arabinose- and galactose-containing side chains while galacturonic acid residues can be partially esterified by methanol on the carboxyl group and by acetyl

on the secondary hydroxyls². Pectin is poorly digested in the small intestine but is fermented by bacteria in the colon, a soluble dietary fibre, exerts physiological effects on the gastrointestinal tract such leading to the production of short chain fatty acids³.

D-Galacturonic acid, an oxidized form of D-galactose, the main monomer of pectin, is an attractive substrate for bioconversions, since pectin-rich biomass is abundantly available and pectin is easily hydrolyzed⁴. The backbone of pectin is composed of 1-4 linked α -D-galactosyluronic acid residues, that comprise 60-70% of total pectic polysaccharides amount⁵.

Galacturonic acid and derivatives can be utilised in food industry (as acidic agents),

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chemical industry (as washing powder agent and nonionic or anionic biodegradable surfactants) and pharmaceutical of industry (for production of vitamin C)⁶. It is also potentially an important carbon source for microorganisms living on plant material, either as a saprotroph or a pathogen⁷. The difference in fermentation pattern between some polysaccharides and oligosaccharides has been also reported for dextran and oligodextrans and various glucose oligosaccharides of different degrees of polymerization⁸. Pectin-derived oligosaccharides (POS) have been proposed as excellent candidates for new-generation prebiotics⁹ which leads us therefore to study the bifidogenic properties of galacturonic acid as via their in vitro fermentation by some gut human bacteria strains

MATERIALS AND METHODS

All chemicals were purchased from Fluka Sigma-Aldrich. D-Galacturonic acid was supplied by Fluk Biochemika, D-glucose was obtained from (Cheminova International, Madrid, Spain), all microbiological culture media were obtained from Algerian Pasteur Institute.

Lactic bacteria, *Lactobacillus reuteri* was isolated from fresh fecal sample on Man, Rogosa and Sharpe (MRS) 24 h at 37°C. *Streptococcus thermophilus* from commercial yogurt on M17 medium 24 h at 40°C and further characterized for their potentials of gastric acidity and bile resistance; antagonistic activity, adhesion, autoaggregation and coaggregation. The pathogenic bacteria strain; *Staphylococcus aureus* was procured from microbiological teaching hospital laboratory (Algiers, Algeria), *Listeria monocytogenes* ATCC 7644 purchased from Algerian Pasteur Institute.

Bacteria identification and characterisation

The identification work was done according to the methods described in Bergeys Manual of determinative bacteriology 9th edition. The probiotic candidate *Lactobacillus reuteri*, *Streptococcus thermophilus* were subjected to a preliminary identification on the basis of Gram staining and catalase reaction then confirmed by carrying out an API 50 system test (BioMérieux). Biochemical identification of *Staphylococcus aureus* was done after confirmation of Gram reaction and catalase test, production of DNase,

coagulase test; antibiotic sensitivity test to novobiocin. Simulated gastric juice and bile solution, at different concentrations on bile and pH, were used to determine the susceptibility of probiotic candidate as described by Both and al¹⁰.

Fermentation procedure

The in vitro digestion medium of galacturonic acid was validated and optimised for mimicking the physiological conditions of upper intestine and microbial conversions in the colon. The best media was the one proposed by Olan-Martin and al (2000)¹¹. The basal culture medium containing 2 g/liter peptone water, 2 g/liter yeast extract, 0.1 g/liter NaCl, 0.04 g/liter K₂HPO₄, 0.04 g/liter KH₂PO₄, 0.01 g/liter MgSO₄·7H₂O, 0.01 g/liter CaCl₂·2H₂O, 2 g/liter NaHCO₃, 0.5 g/liter L-cysteine HCl, 0.5 g/liter bile salts, 10 µl vitamin K1, 2 ml Tween 80, and 10 ml hemin. The medium was adjusted to pH 6.8 then autoclaved at 121°C for 15 min.

Bacterial growth in the presence of galacturonic acid

Growth kinetics of the four bacterial strains were carried out in this media containing an optimal concentration of 1% glucose as control substrate and galacturonic acid as treatment (after optimization of the substrate content 0.1, 0.5, 1 and 5%) for an incubation period of 48 hrs at 37°C. Growth was measured spectrophotometry every 2 hrs at 600 nm up to 48 h and pH was measured at each time-point. A sample (2 ml) was taken from the inoculum to determine microbial counts and pH at time 0.

In vitro evaluation of galacturonic acid on EPS production by probiotic strains

The EPS extraction assay was performed by the method of Pawar *et al*¹². This medium is used as a base for exopolysaccharides production, it was consisting of the following components (g/l): peptone 10, meat extracts 3, sodium chloride 5. The pH was adjusted to 6.5, then autoclaved at 121°C for 15 min, 50 sucrose, 50 mannose and 50 galacturonic acid as alternative sole carbon source were added by microfiltration (millipore filter 0.22 µm). The flasks were incubated on a rotary shaker at room temperature for 72 hrs. After incubation, the cultures were heated at 100 °C for 15 min, and bacterial cells were removed by centrifugation, were harvested by centrifugation 5000 rpm for 20 min. The recovered supernatant was filtered

through a millipore filter (0.22 μm) then two volumes of cold isopropanol (4°C) were added into it and stored overnight at 4°C. Precipitated material was collected by centrifugation 20 min at 5000, after discarding the supernatant, the precipitate of EPS was washed separately with ethanol and the pellets were dried at 100 °C.

Statistical Study

The kinetics of growth and exopolysaccharids production were realized on duplicate, then for the growth test an obtained difference of 1 Log CFU/mL is considered as significant.

RESULTS AND DISCUSSION

Bacteria identification and characterisation

The results have demonstrated that characterized lactic strains possess useful

probiotic properties. Indeed, they have proved a good heat resistance (at a temperature of 65°C); an important quality when certain technological treatments. Probiotics candidates were not only tolerant to acid (to a pH of 2) but more capable of growth and multiplication; two particularly important parameters to late to overcome the gastric barrier. Resistance to bile salts is an important selection criterion and are powerful antimicrobial agents at low concentrations.

The studied probiotic candidates have revealed other hand a significant inhibitory activity with regard to the target bacteria (*S. aureus* and *Listeria monocytogenes*) and a great ability to membership, to auto-aggregation; essential qualities for the colonization of the intestine and competition with pathogenic bacteria on membership sites.

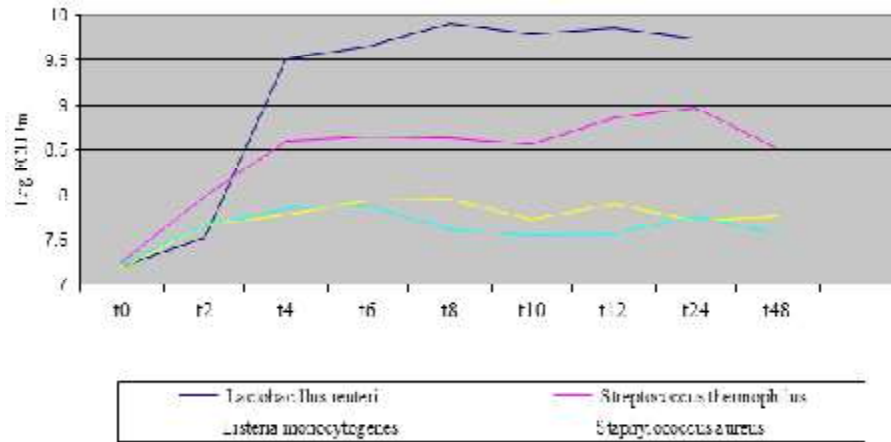


Fig. 1. Growth and fermentation kinetics of studied bacteria strain on galacturonic acid at 1%

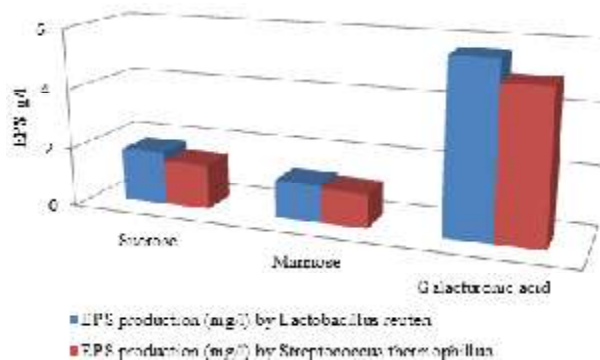


Fig. 2. Probiotics candidates EPS production on galacturonic acid

Bacterial growth in the presence of galacturonic acid

After several tests of different culture media *in vitro*, that proposed by Olano-Martin and al (2000) was the most appropriate. After we optimized the galacturonic acid concentration as the sole carbon source (0.1-0.5-0.8 and 1%) of D-galacturonic acid. The best growth kinetic was with the higher concentration 1%. Which correlate with the works of Zhang and his collaborators. Indeed, several genes involved in pectin decomposition and D-Galacturonic catabolism are inducible *in vitro* by D-galacturonic acid¹³. The prebiotic candidate was tested for their effect on proliferation of the gut pathogen *Listeria monocytogenes*, *Staphylococcus aureus* and two probiotics. The results of this study show a selective stimulation of potential probiotic bacteria, in fact, *Lactobacillus reuteri* and *Streptococcus thermophilus* strains represent at time 4 hrs h 9.58 Log FCU/ml and 8.63 FCU/ml respectively (fig1). However the two target strains represent a significantly lower growth including *Listeria monocytogenes* 7.78 Log FCU / ml and *Staphylococcus aureus* 7.85 Log FCU / ml. The monosaccharide D-galacturonic acid is the ultimate hydrolytic product released by the joint action of endo-PGs and exo-PGs.

This results concord with several recent clinical studies Olano-Martin et al. who observed that pectin and its galacturonic acid stimulated the growth of certain strains of *Bifidobacteria* and *Lactobacillus* *in vitro*. These bacteria are considered to be directly related to the health of the large intestine and their concentrations depict a healthy microflora population.

In vitro evaluation of galacturonic acid on EPS production by probiotic strains

The exopolysaccharide are economically important because they can confer beneficial health effects. Several authors have stated that medium composition specially carbon source, is an important parameter in EPS biosynthesis¹⁴.

In fact, Our results show that irrespective of the strain studied, dependent variations in the carbon source are the most important. Indeed, the best production was observed when bacteria were grown on galacturonic acid, three times more than the presence of sucrose. 5,68 g/l and 4.98 g/l respectively by *Lactobacillus reuteri* and

Streptococcus thermophilus While with mannose, the two bacterial strains produce significantly less EPS, less than 1.25 g/l.

CONCLUSION

In conclusion, the obtained *in vitro* results indicated that galacturonic acid has revealed a significant prebiotic effect by improving the growth of probiotic bacteria *Lactobacillus reuteri* and strongly inhibited *Listeria monocytogenes* and *Staphylococcus aureus* growth; In addition the production of EPS were improved in the presence of this interesting probiotic and the production was estimated for a long phase growth. Galacturonic acid is promising to influence the potential of the microbiota normally associated to the host to modulate a positive response for the human health. Further studies must be conducted in placebo-controlled dietary intervention trials in humans to obtain the final proof that these candidate prebiotic compounds can be really classified as prebiotic.

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