JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2016. Vol. **10**(3), p. 1865-1869

In vitro Fermentation and Bifidogenic Potential of Galacturonic Acid

Elkeurti Khadidja Nadia and Sahmoune Mohamed Nasser

Soft Technology Laboratory, Physical and Chemical Upgrading of Biological Materials and Biodiversity. University M'Hamed Bougara, Boumerdes,
Quoted Frantz Fanon, 35000 Boumerdes, Algeria.
Department of Cellular and Molecular Biology, USTHB Algiers.

(Received: 17 June 2016; accepted: 26 July 2016)

The first aim of our study was to screen some humain 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 prebiotic influenced the auto-aggregation of probiotic candidates, in second time the eventuel effect of D-galacturonic acid on EPS production of the two probiotic candidate on various carbon source. Our resullts 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 prebiotic 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 significantally the probiotic EPS production and i twas 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 plants[1]. 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- l -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 hydroxyls[2]. 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 acids[3].

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 hydrolyzed[4]. The backbone of pectin is composed of 1-4 linked α-D-galactosyluronic acid residues, that comprise 60-70% of total pectic polysaccharides amount[5].

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

* To whom all correspondence should be addressed.
E-mail: kmelkeurti@yahoo.fr

chemical industry (as washing powder agent and nonionic or anionic biodegradable surfactants) and pharmaceutic 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 pathogen[7]. 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 polymerization[8] Pectin-derived oligosaccharides (POS) have been proposed as excellent candidates for new-generation prebiotics[9] 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 fersh 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 mthe 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 al[10].

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 Olano-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 K_2HPO_4, 0.04 g/liter KH_2PO_4, 0.01 g/liter $MgSO_4 \cdot 7H_2O$, 0.01 g/liter $CaCl_2 \cdot 2H_2O$, 2 g/liter $NaHCO_3$, 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 spectophotometry 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 exoplysaccharides 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^{0}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 at100 ^{0}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.

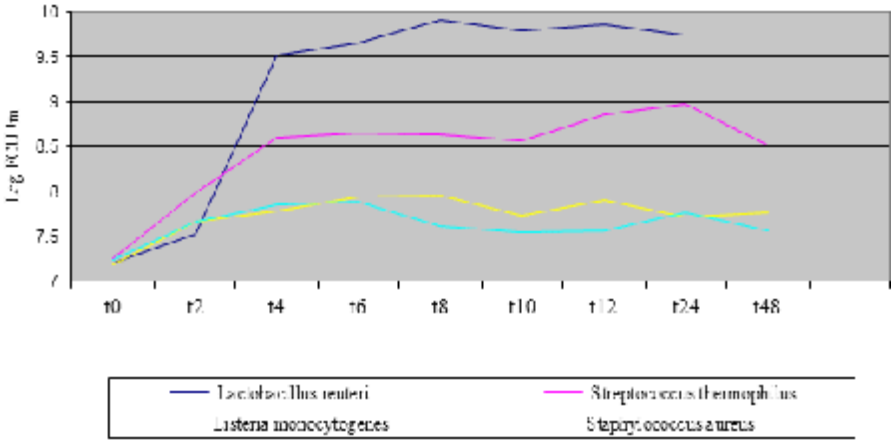

Fig. 1. Growth and fermentation kinetics of studied bacteria strain on galacturonic acid at 1%

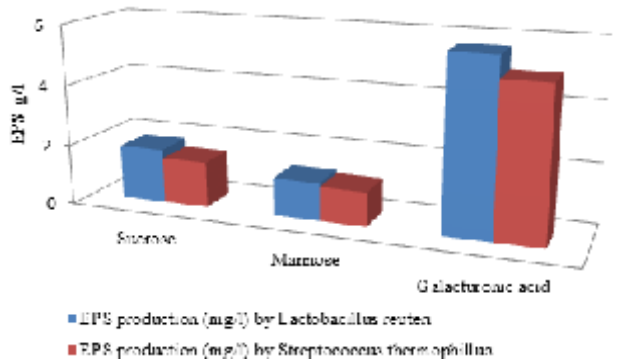

Fig. 2. Probiotics candidates EPS production on galacturonic acid

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%. Witch corhelate 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 acid[13]. 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 biosynthesis[14].

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 resectively by *Lactobacillus reuteri* and *Streptococcus thermophillus* 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.

REFERENCES

1. Dey.P.M ; Harborne J.B : Plant Biochemistry. 1997, Academic press. pp. 215-216
2. Ralet Marie-Christine ; Crépeau Marie-Jeanne ; Buchholt Hans-Christian ; Thibault Jean-François : Polyelectrolyte behaviour and calcium binding properties of sugar beet pectins differing in their degrees of methylation and acetylation. LWT - *Food Science and Technology*. 2011, **44,** pp. 1687-1696.
3. Maazullah Khan ; Nizakat Bibi and Aurang Zeb : Agriculture, Optimization of Process Conditions for Pectin Extraction from Citrus Peel, *Science, Technology and Development* : 2015, **34**, pp. 9-15.
4. Kuivanen J ; Mojzita D ; Wang Y ; Hilditch S ; Penttilä M ; Richard P ; Wiebe MG : Engineering Filamentous Fungi for Conversion of d-Galacturonic Acid to l-Galactonic Acid , *Appl Environ Microbiol*, 2012, **24**, 8676–8683.
5. Molnár Eszter ; Nemestóthy Nándor ; Bélafi-Bakó Katalin : Utilisation of bipolar electrodialysis for recovery of galacturonic acid, Desalination, 2010, 250, pp. 1128–1131.
6. Molnar Eszter ; Eszterle Matild ; Kiss Kornelia ; Nemestothy Nandor ; Fekete Jeno ; Belafi-Bako Katalin : Utilization of electrodialysis for galacturonic acid recovery. Desalination, 2009.

241, pp. 81-85.

7. Zhang Lisha ; Thiewes Harry ; Kan Jan A.L.van : The d-galacturonic acid catabolic pathway in *Botrytis cinerea*. *Fungal Genetics and Biology*, 2011. **48**, 990–997.
8. Olano-Martin. E ; Gibson. G.R and Rastall R.A : Comparison of the in vitro bifidogenic properties of pectins and pectic oligosaccharides. *Journal of Applied Microbiology*. 2002, **93**, 505–511.
9. Gullón Beatriz ; Gullón Patricia ; Sanz Yolanda ; Alonso José Luis ; Parajó J.C : Prebiotic potential of a refined product containing pectic oligosaccharides. LWT - *Food Science and Technology*. 2011, **44**, 1687–1696.
10. Both emese ; györgy éva ; Kibédi-szabó casaba ; Tamás éva ; Beata ábrahám, Miklóssy ildiko, Lányi szabols : acid and bile tolerance, adhesion to epithelial cells of probiotic microorganisms. *U.P.B. Sci. Bull*. 2010, **72**, 37- 44.
11. Olano-Martin E ; Moumtzouris K. C ; Gibson G. R and Rastall R. A: In vitro fermentability of dextran oligodextran and maltodextrinby human gut bacteria. *British journal of nutrition*. 2000, **83**, 247 – 255.
12. Pawar Sunil T ; Bhosale Amarsinh A ; Gawade Trishala B. and Nale Tejswini R : Isolation, screening and optimization of exopolysaccharide producing bacterium from saline soil. *J. Microbiol. Biotech. Res*. 2013, **3**, 24-31.
13. Zhang Lisha ; Hua Chenlei ; Stassen Joost H.M ; Chatterjee Sayantani ; Cornelissen Maxim ; Kan Jan A.L. van : Genome-wide analysis of pectate-induced gene expression in*Botrytis cinerea*: Identification and functional analysis of putative D-galacturonate transporters. *Fungal Genetics and Biology*. 2014. **72**, 182–191.
14. Bennama Rabha ; Ladero Victor ; Alvarez Miguel; Fernández María and Bensoltane Ahmed : Effect of fermentation conditions (culture media and incubation temperature) on exopolysaccharide production by Streptococcus thermophilus BN1. *Environment and Chemistry*. 2011. **24**, 433-437.