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# The impact of distillation process on prebiotic activity of different oligosaccharidic fractions extracted from grape seeds

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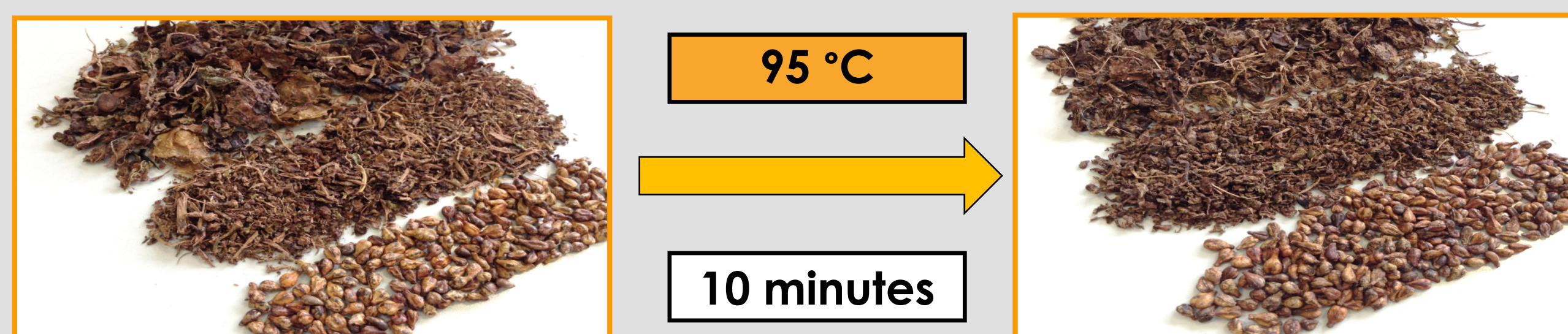
## OVERVIEW AND AIM OF THE WORK

Grape pomace, a remnant of the wine making process, is one of the most important residues of the wine industry. It consists of different amounts of grape, skin, pulp, seeds, and, if not removed, stems. Some industrial uses currently under investigation for wine pomace waste include use as animal feed, as possible nutritive ingredients for value added products, in the production of citric acid, and the use of anthocyanins from grape skins as colorants (Bordiga et al., 2013). Spirits from fermented grape pomace are very popular in Mediterranean countries. Several countries produce traditional distilled alcoholic beverages from different raw materials. Grappa is a typical Italian spirit of commercial, cultural and historical importance, which is obtained by processing grape pomace or marcs from must or wine at the end of alcoholic fermentation. The high concentrations of bioactive compounds and the variation of their concentrations among different parts of grape pomace show the importance of analyzing in depth this winemaking by-product as a good and economically viable source of natural molecules (Bordiga et al., 2011). Despite the degradation caused by the drastic conditions of the distillation process, many interesting compounds still exist in this spent matrix (Bordiga et al., 2015). Together with a high content in phenols, grape seeds also contain a certain amount of oligosaccharides (Bordiga, 2015). One of the most well-demonstrated actions of oligosaccharides is the prebiotic activity.

**Aims of this study was to evaluate different oligosaccharidic fractions, extracted from PRE and POST distilled grape seed pomace, as potential functional ingredients with prebiotic activity toward well-known probiotic bacteria, allowing to improve the growth during in vitro fermentation.**

## EXPERIMENTAL

### DISTILLATION PROCESS



### EXTRACTION PROCESS

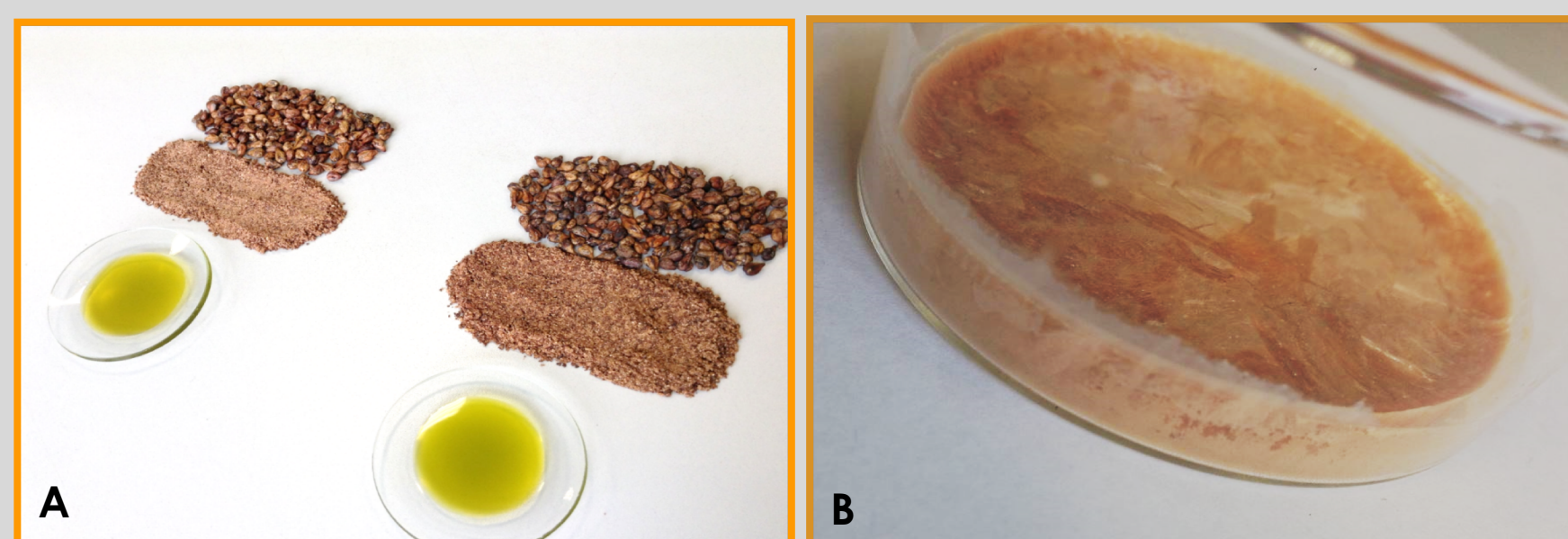


Figure 1 : A) ground defatted seeds, PRE (left) and POST (right); B) example of a lyophilized oligosaccharidic fraction.

Grape seeds were ground to a powder using liquid N<sub>2</sub>. Seeds powder was extracted with Soxhlet apparatus using hexane for 9 h to remove oil (Figure 1A). Subsequently, ground defatted seeds were extracted using EtOH/water (85:15, v/v) to obtain crude extract. Proper oligosaccharides purification has been realized by using an appropriate multistep solid-phase extraction (using C-18 cartridge followed by carbograph cartridge) (Figure 1B). Elution by the latter cartridge was carried out with H<sub>2</sub>O to obtain the first fractions (PreH<sub>2</sub>O and PostH<sub>2</sub>O). The second oligosaccharidic fractions were then eluted with a 60:40 deionized H<sub>2</sub>O/ACN solution containing 0.1% trifluoroacetic acid (Pre40 and Post40). Finally, the third fractions released with a 20:80 deionized H<sub>2</sub>O/ACN solution containing 0.05% trifluoroacetic acid (Pre80 and Post80).

## RESULTS AND DISCUSSION

### CHARACTERIZATION OF OLIGOSACCHARIDES

The glycosyl residue composition determined by GC after acidic hydrolysis of oligosaccharides is summarized in Table 1. Galactose (Gal), arabinose (Ara), mannose (Man) and glucose (Glc) represent the major constituents of oligosaccharides from grape seeds. The composition of the PRE and POST extracts is very similar.

% Mol	PREH <sub>2</sub> O	POSTH <sub>2</sub> O	PRE40	POST40	PRE80	POST80
2-O-MeFuc <sup>a</sup>				1.1		
Rha		2.9	11.6			
Fuc			1.4			
Ara	84.4	82.5	73.4	62.1	24.4	23.1
Xyl		1.6	Tr.	2.8		
Api				1.2		
Man	2.8	3.2	2.6	4.0	11.3	19.6
Gal	2.4	4.5	12.6	10.2	10.5	13.5
Glc	10.4	8.2	7.8	5.0	53.8	43.8

**Table 1.** Glycosyl residue composition (% Mol) of Oligosaccharides from PreH<sub>2</sub>O, PostH<sub>2</sub>O, Pre40, Post40, Pre80 and Post80 fractions after purification by high-resolution size exclusion chromatography on a Superdex Peptide column. <sup>a</sup>2-O-MeFuc, 2-O-CH<sub>3</sub>-fucose; Rha, rhamnose; Fuc, fucose; 2-O-MeXyl, 2-O-CH<sub>3</sub>-xylose; Ara, arabinose; Api, apiose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose.

Chemical characterization of the complex oligosaccharides from grape seeds extract was performed using a UPLC-ESI-MS<sup>n</sup> (Qiang et al., 2009). Figure 2 (A to F) show the total ion profiles obtained by UPLC-MS analysis on a Nucleodur HILIC column of Oligosaccharides from PreH<sub>2</sub>O (Fig 1-A), PostH<sub>2</sub>O (Fig 1-B), Pre40 (Fig 1-C), Post40 (Fig 1-D), Pre80 (Fig 1-E) and Post80 (Fig 1-F) fractions. Analysis of the full-scan mass spectra of Oligosaccharides from PreH<sub>2</sub>O and PostH<sub>2</sub>O, Pre40 and Post40, Pre80 and Post80 fractions (Figure 3) showed a multitude of masses between m/z 400 and m/z 1600 Da. The full-scan mass spectrum analysis also shows the presence of mono- and di-charged ions.

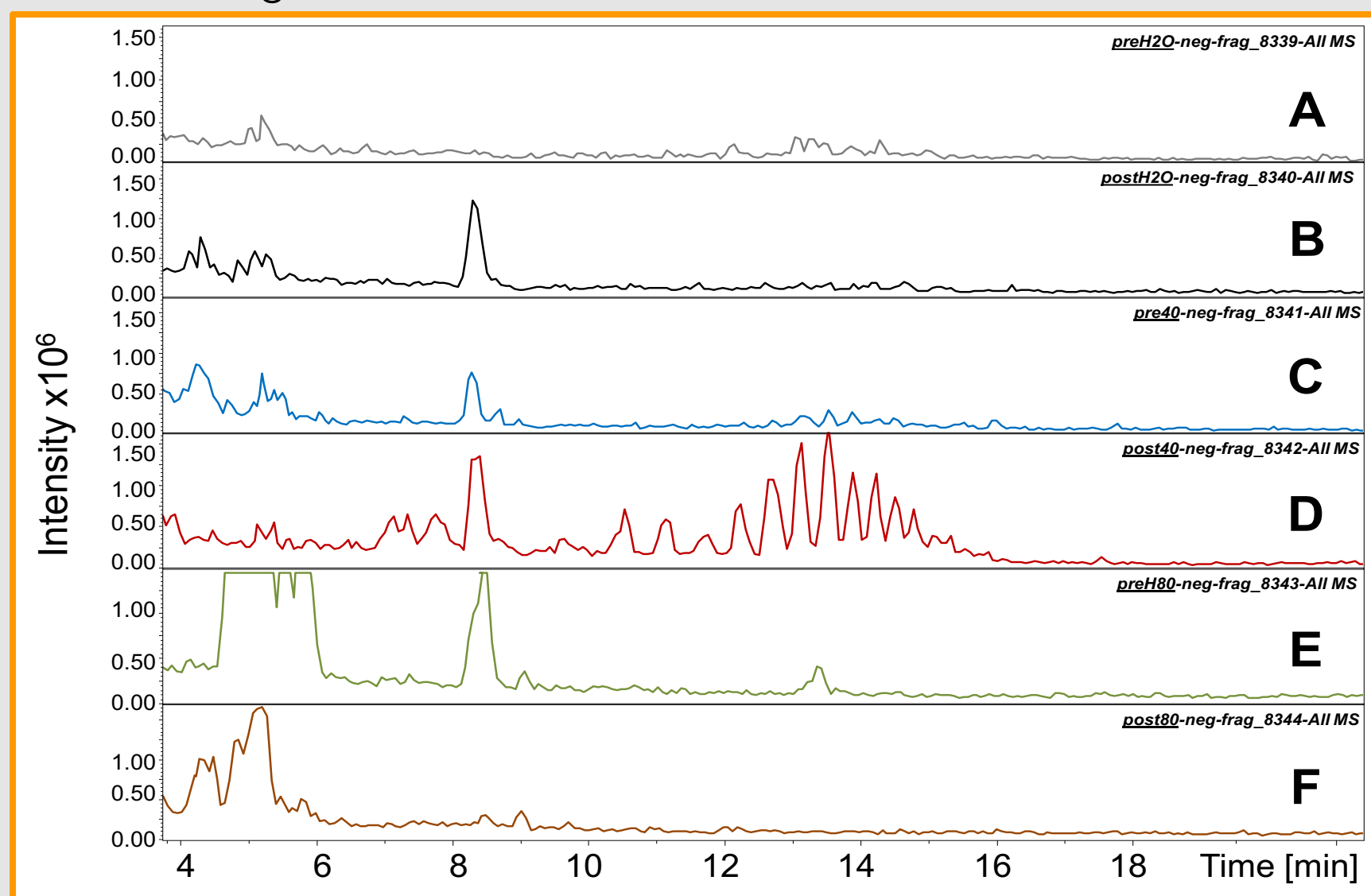


Figure 2 : Total ion profiles obtained by UPLC-MS analysis on a Nucleodur HILIC column of Oligosaccharides from PreH<sub>2</sub>O (A), PostH<sub>2</sub>O (B), Pre40 (C), Post40 (D), Pre80 (E) and Post80 (F) fractions after purification by SEC on a Superdex Peptide column.

Complex oligosaccharides from grape seeds extract purified by size exclusion chromatography consist mainly on a neutral oligosaccharides rich in arabinose and certainly in glucose. These neutral oligosaccharides identified were really similar to those identify from Carignan wines (oligo-arabinans, oligorhamno-arabinans, and different arabinorhamnogalacturonan-oligosaccharides, Doco et al., 2015) and from Grignolino and Chardonnay wines (hexose-oligosaccharides, oligoxyloglucans, and oligoarabinogalactans, Bordiga et al., 2012). They represent the degraded structures of polysaccharides originating from the cell wall degraded by enzyme activities.

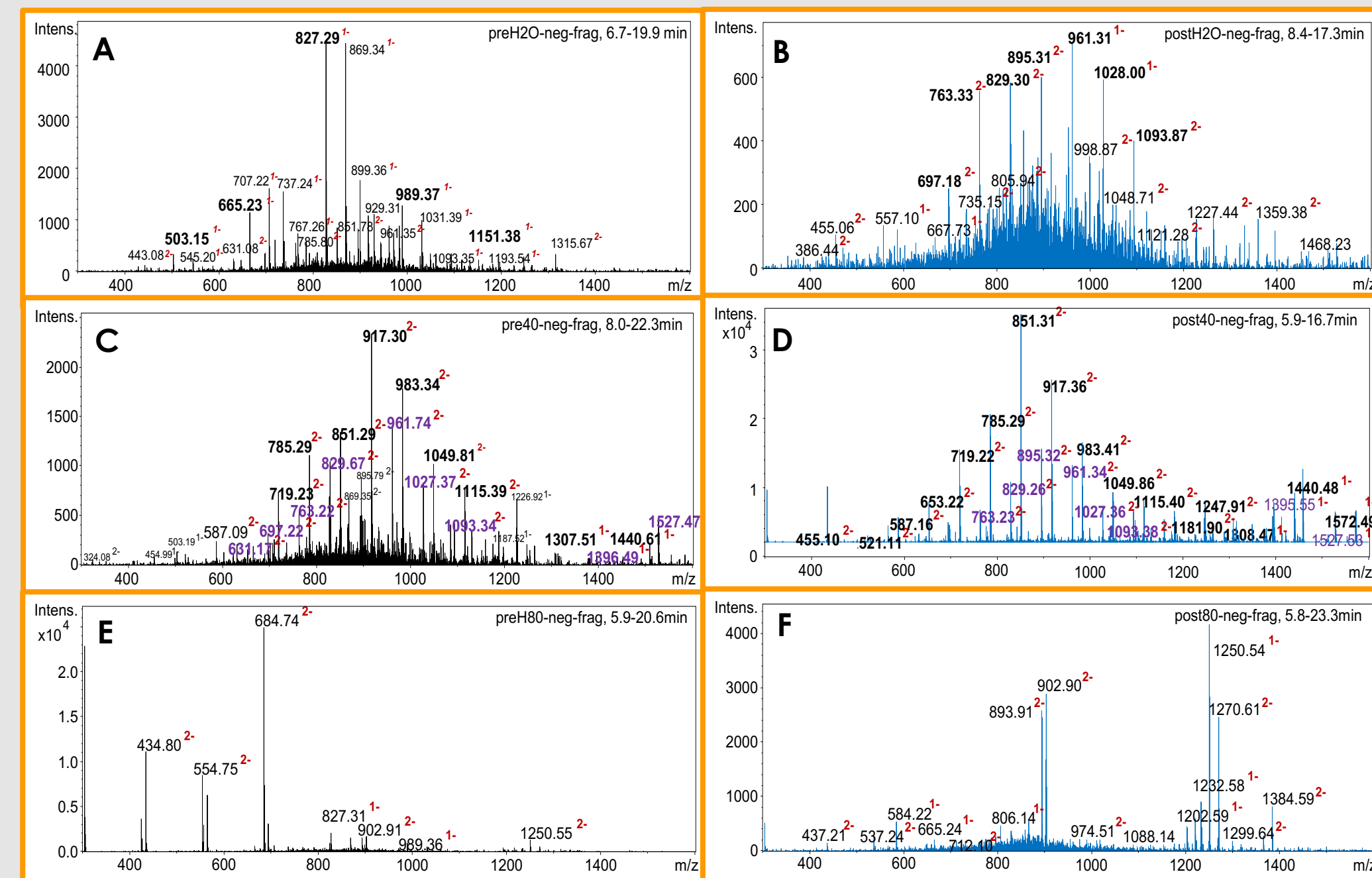


Figure 3 : Full-scan mass spectrum of Oligosaccharides from PreH<sub>2</sub>O (A), PostH<sub>2</sub>O (B), Pre40 (C), Post40 (D), Pre80 (E) and Post80 (F) fractions after purification by SEC on a Superdex Peptide column.

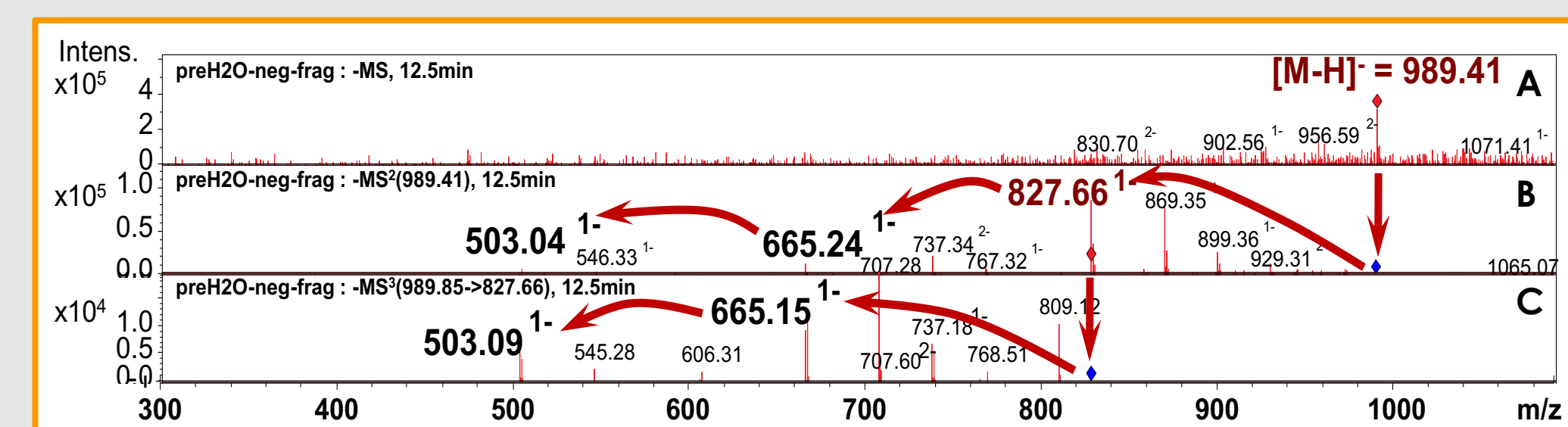
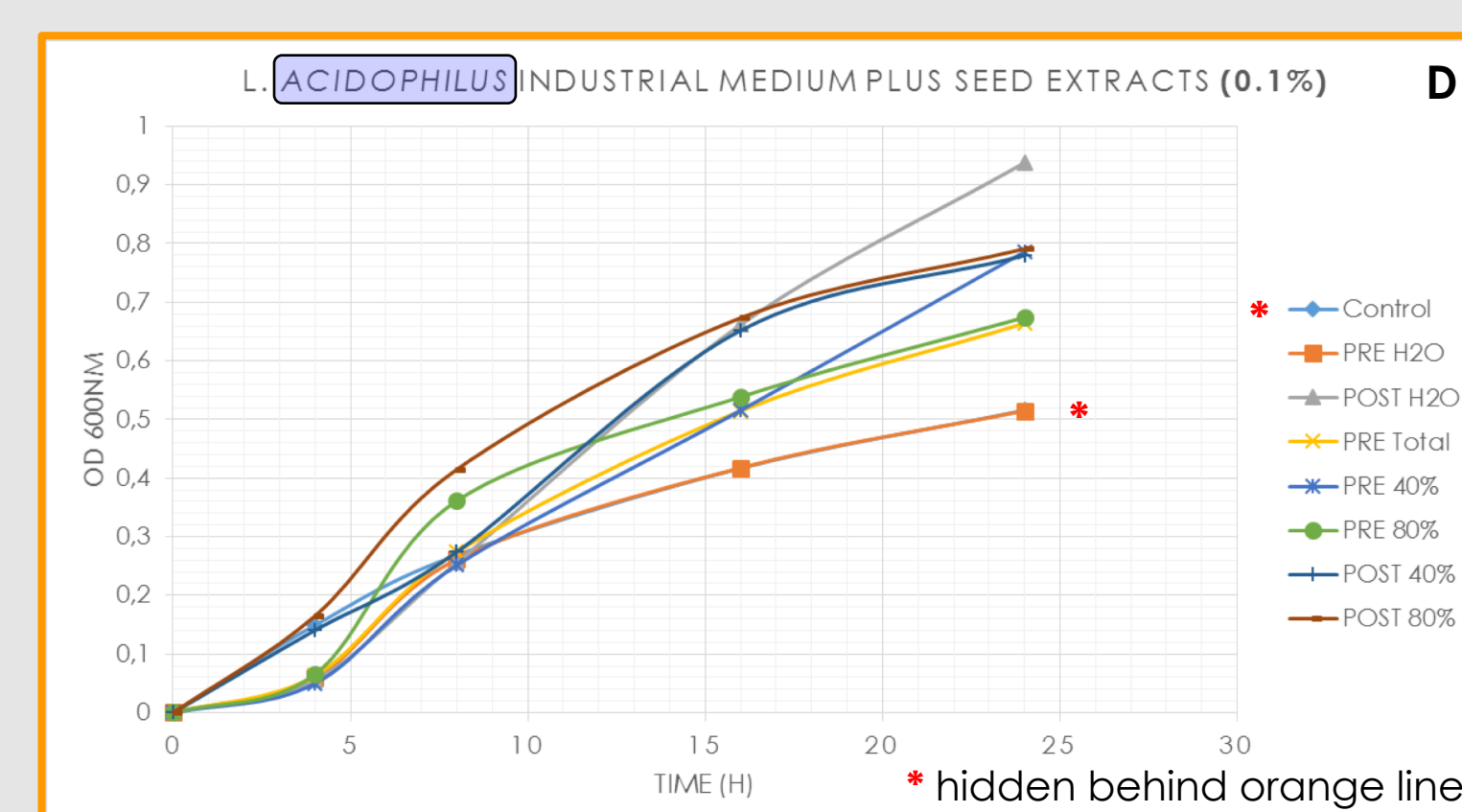
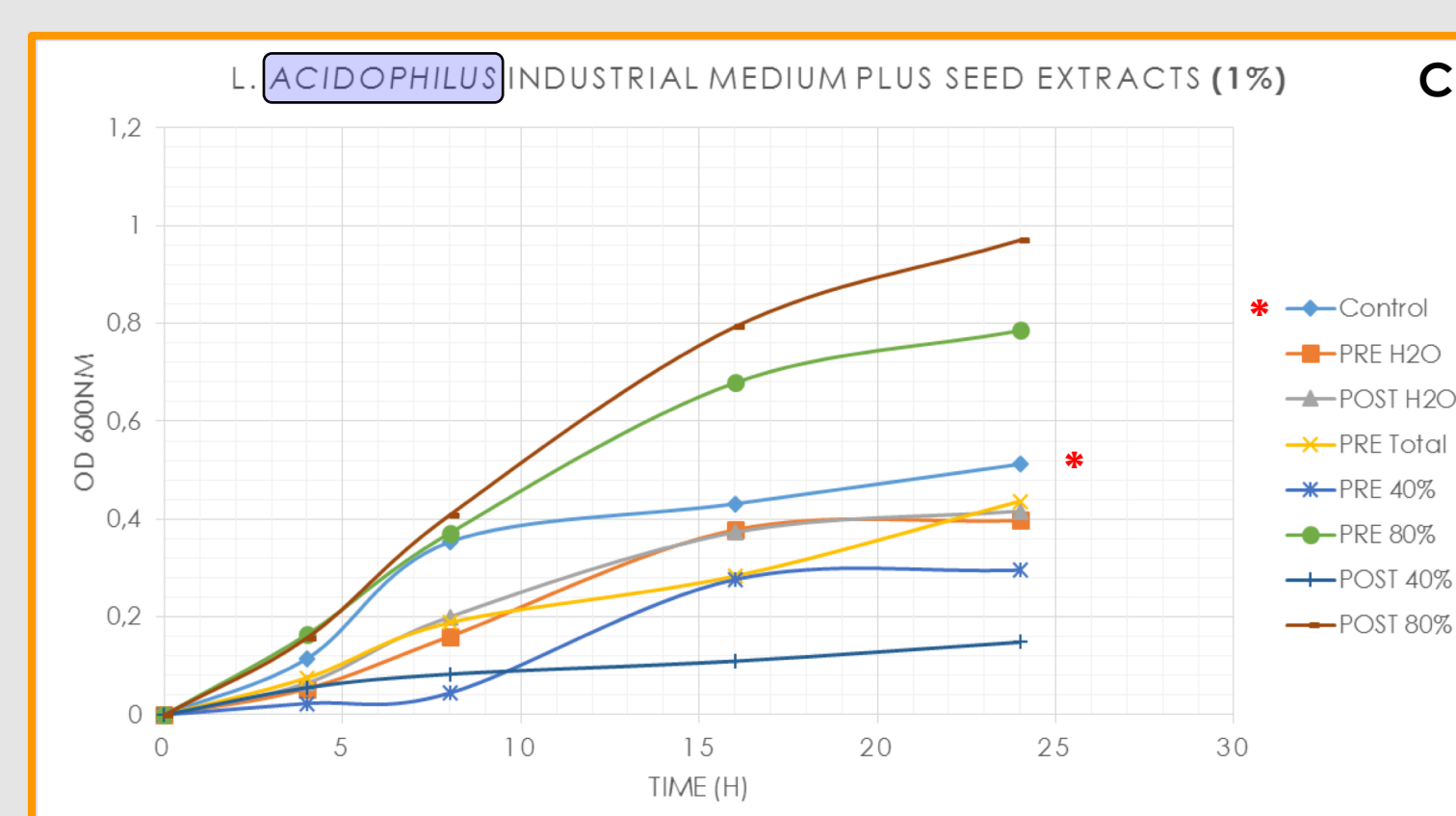
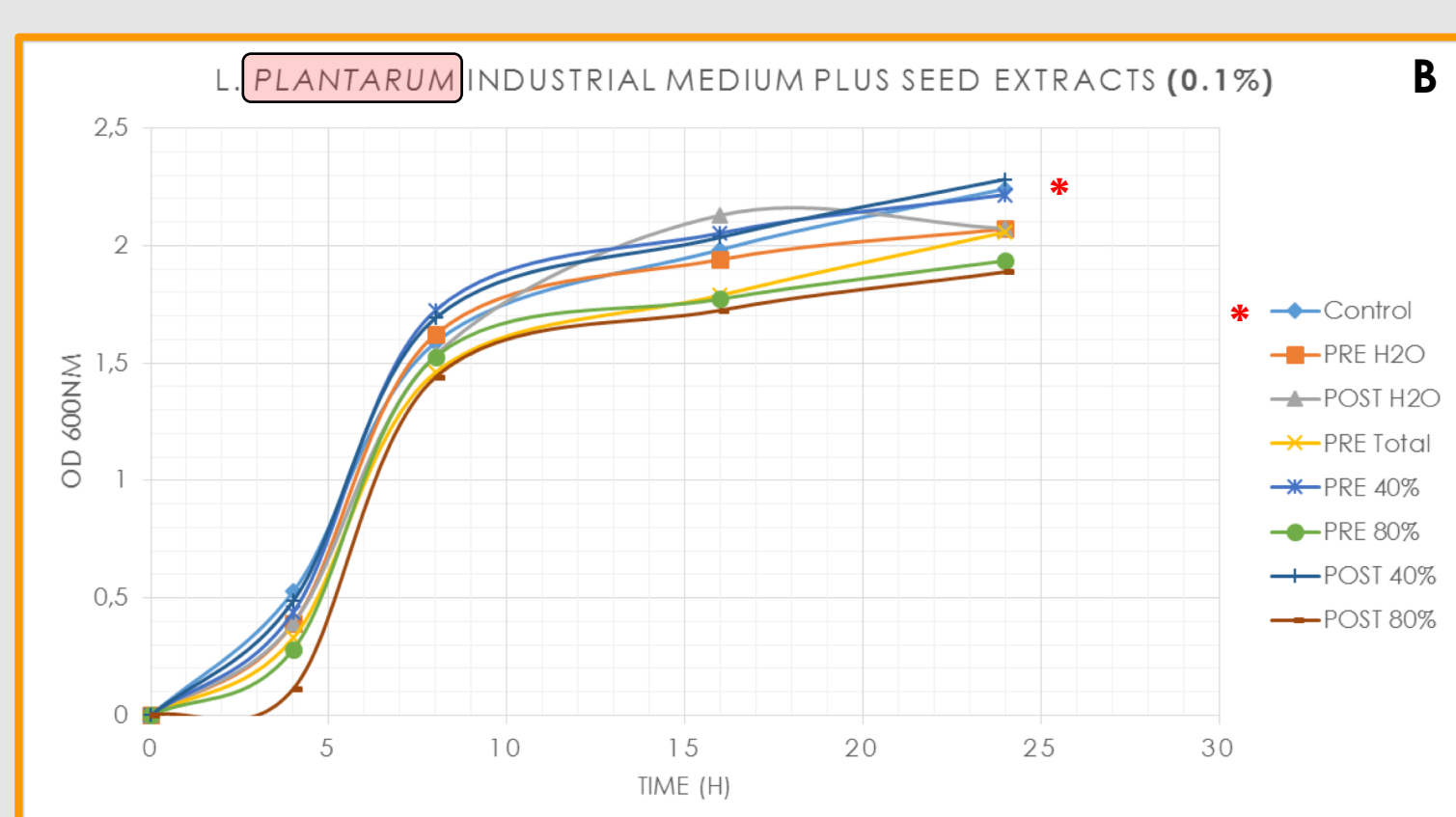
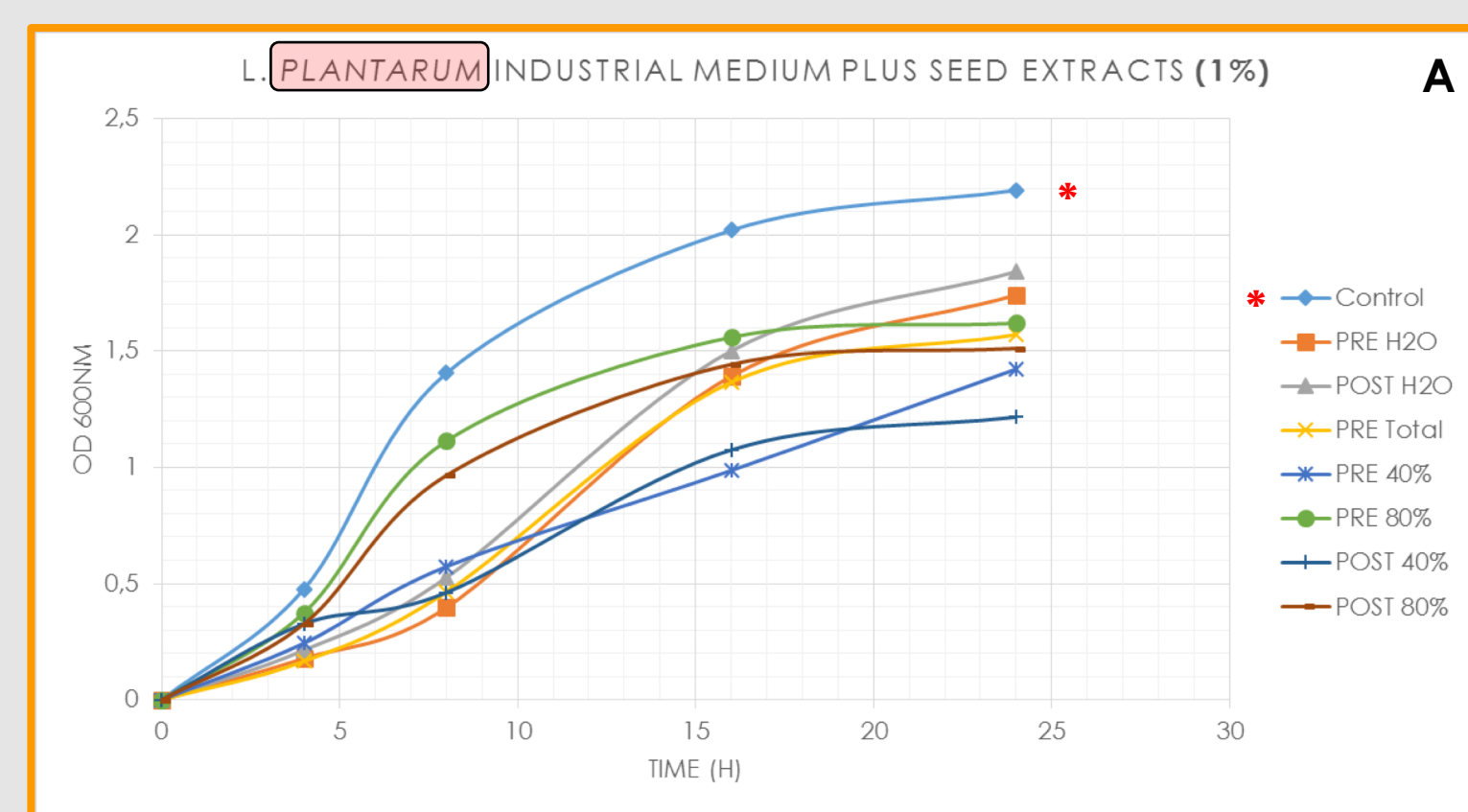


Figure 4 : Spectra of MS3 fragmentation by ESI-TI in negative mode of ion parent at m/z 989.41 (A), MS2 spectrum of the ion at m/z 989.41 [M-H]<sup>-</sup>, (B), MS3 spectrum of the ion at m/z 826.66 [M-H]<sup>-</sup> from the m/z 989.41 [M-H]<sup>-</sup>. → Loss of a fragment of m/z 162 Da.

In Figure 3A, the full-scan mass spectra of oligosaccharides from PreH<sub>2</sub>O, a series of ions was observed at m/z 503.15, 665.23, 827.29, 989.37 and 1151.38, with a difference of 162 Da between the different ions. The ions of the series correspond to oligosaccharide molecules as single deprotonated [M-H]<sup>-</sup> ions.

The difference find between the ions of the series corresponds to the presence of a hexose (m/z 162 Da), likely a glucose residue. Figure 4A shows, as an example, the MS<sup>n</sup> spectra of the [M-H]<sup>-</sup> ion at m/z 989.41 obtained from the oligosaccharides of PreH<sub>2</sub>O fraction. The fragment ions observed in the MS<sup>n</sup> spectra are usually named according to the nomenclature of Domon and Costello (1988). MS<sup>n</sup> spectra and the fragment ions obtained of deprotonated oligosaccharides can be read from "right to left". The MS<sup>2</sup> fragmentation (figure 4B) of the parent ion at m/z 989.41 showed the presence of a fragment ion at m/z 827.66, due to the loss of a hexose residue (m/z 162 Da). This fragment ion at m/z 827.66 loses a second hexose residue to give the fragment ion at m/z 665.24, and a successive loss of hexose residue yield fragment ion at m/z 503.04. MS<sup>3</sup> fragmentation (figure 4C) of the fragment ion at m/z 827.66 showed the presence of fragment ions arising from successive losses of hexose residues, fragment ions at m/z 665.15 and m/z 503.09.

## GROWTH TEST (IN VITRO)



The probiotic strains *L. plantarum* P17630 and *L. acidophilus* P18806 were cultured in an industrial medium (specifically optimized for each strain) added with two different concentrations of oligosaccharidic fractions (OF) (1.0% and 0.1%, respectively). Results have been compared to control, representing the growth of the strain using exclusively their specific industrial medium. Considering the *L. plantarum* P17630, any significant additional improvement on bacterial growth was observed when the OF were added (Figures 5A and 5B). Especially when the addition was 1.0%, all the results obtained with the different OF were significantly lower if compared to control. Results (substantially) comparable have been obtained instead when the addition was 0.1%. Conversely, considering the *L. acidophilus* P18806, novel and interesting results have been obtained. Post80 and Pre80 showed significantly higher values when the addition was 1.0% (Figure 5C). However, the best performance has been obtained when the addition of oligosaccharidic fractions was 0.1%. All the fractions (except for PreH<sub>2</sub>O) showed significantly higher values during growth test. These results demonstrate that, under these conditions, the addition of these bioactive compounds are able to generate a sort of "booster effect". However, looking at the results, it appears clear that each strain responds differently to the addition of these compounds. A possible explanation for this effect could be correlated to the high specie-specificity (and strain-specificity) of each carbohydrate substrate, because of the need of a specific and appropriate enzymatic pattern for the linkage degradation typical for each single microorganism, as well as the existence of specific pathways for the uptake of the substrate from the medium.

## CONCLUSIONS

The final conclusion from this study is that despite the degradation caused by the drastic conditions of the distillation process, many interesting, valuable compounds are found in the extract from the waste of this process, which make it a useful matter for a better exploitation than as a heating source. Oligosaccharides still contained (yield of about 1% from degreased grape seeds) may be considered a novel "functional ingredient" with potential prebiotic activity towards *L. plantarum* P17630 and *L. acidophilus* P18806, allowing to improve the growth during in vitro fermentation.

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