



UNIVERSITÀ POLITECNICA DELLE MARCHE  
Repository ISTITUZIONALE

A spectroscopic study on orthodontic aligners: First evidence of secondary microplastic detachment after seven days of artificial saliva exposure

This is the peer reviewed version of the following article:

*Original*

A spectroscopic study on orthodontic aligners: First evidence of secondary microplastic detachment after seven days of artificial saliva exposure / Quinzi, Vincenzo; Orilisi, Giulia; Vitiello, Flavia; Notarstefano, Valentina; Marzo, Giuseppe; Orsini, Giovanna. - In: SCIENCE OF THE TOTAL ENVIRONMENT. - ISSN 0048-9697. - 866:(2023). [10.1016/j.scitotenv.2022.161356]

*Availability:*

This version is available at: 11566/309726 since: 2024-04-12T11:21:26Z

*Publisher:*

*Published*

DOI:10.1016/j.scitotenv.2022.161356

*Terms of use:*

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. The use of copyrighted works requires the consent of the rights' holder (author or publisher). Works made available under a Creative Commons license or a Publisher's custom-made license can be used according to the terms and conditions contained therein. See editor's website for further information and terms and conditions.

This item was downloaded from IRIS Università Politecnica delle Marche (<https://iris.univpm.it>). When citing, please refer to the published version.

(Article begins on next page)

1 A spectroscopic study on orthodontic aligners: first evidence of secondary  
2 microplastic detachment after seven days of artificial saliva exposure

3  
4 Vincenzo Quinzi <sup>a§</sup>, Giulia Orilisi <sup>b§</sup>, Flavia Vitiello <sup>b</sup>, Valentina Notarstefano <sup>c</sup>, Giuseppe Marzo <sup>a</sup>,  
5 Giovanna Orsini <sup>b\*</sup>

6  
7 <sup>a</sup> *Department of Health, Life and Environmental Science, University of L'Aquila, Piazza Salvatore*  
8 *Tommasi, 67100 L'Aquila, Italy*

9 <sup>b</sup> *Department of Clinical Sciences and Stomatology, Polytechnic University of Marche, Via Tronto*  
10 *10, 60126 Ancona, Italy*

11 <sup>c</sup> *Department of Life and Environmental Sciences, Polytechnic University of Marche, Via Brecce*  
12 *Bianche, 60131 Ancona, Italy*

13  
14 \*Corresponding author: [g.orsini@staff.univpm.it](mailto:g.orsini@staff.univpm.it); +39 3472483290.

15 § The Authors equally contributed to the manuscript.

16  
17 ABSTRACT

18 Clear orthodontic aligners have recently seen increasing popularity. The thermoplastic materials  
19 present several advantages, even if it is known that all plastic products can be subjected to  
20 environmental and mechanical degradation, leading to the release of microplastics (MPs). Their  
21 ingestion could cause oxidative stress and inflammatory lesions. This study aims to evaluate the  
22 potential detachment of MPs by clear aligners due to mechanical friction simulated with a 7-days  
23 protocol in artificial saliva. The study was performed on orthodontic clear aligners from different  
24 manufacturers: Alleo (**AL**); Flexi Ligner (**FL**); F22 Aligner (**F22**); Invisalign (**INV**); Lineo (**LIN**);  
25 ArcAngel (**ARC**), and Ortobel Aligner (2). For each group, two aligners were immersed in artificial  
26 saliva for 7 days and stirred for 5 hours/day, simulating the physiological teeth mechanical friction.  
27 After 7 days, the artificial saliva was filtered; filters were analyzed by Raman Microspectroscopy  
28 (RMS) and Scanning Electron Microscopy (SEM), respectively to chemically identify the polymeric  
29 matrix and to measure the number and size of the detected MPs. MPs were evaluated in terms of  
30 chemical composition, number, and size. RMS spectra revealed that **AL**, **FL**, **LIN**, **ARC**, and **OR**  
31 aligners were composed by polyethylene terephthalate, while **F22** and **INV** ones by polyurethane.  
32 SEM analysis showed that the highest number of MPs was found in **ARC** and the lowest in **INV**  
33 ( $p < 0.05$ ). As regards MPs' size, no statistically significant difference was found among groups, with

34 most MPs ranging from 5 to 20  $\mu\text{m}$ . Noteworthy, a highly significant correlation ( $p < 0.0001$ ) was  
35 highlighted between the distribution of MPs size and the different typologies of aligners. This in vitro  
36 study highlighted for the first the detachment of MPs from clear aligners due to mechanical friction.  
37 This evidence may represent a great concern in the clinical practice since it could impact human  
38 general health.

39

40 *Keywords:* Clear orthodontic aligners; Microplastics; Raman Microspectroscopy; Scanning Electron  
41 Microscopy.

42

### 43 **1. Introduction**

44 To date, the growing demand for “invisible” orthodontic treatments among both child and adult  
45 patients, led to an upsurge in the development of esthetic and comfortable alternatives to conventional  
46 fixed appliances (Kesling, 1946; Macri et al., 2022). Thanks to the introduction in dentistry of  
47 CAD/CAM technologies, the use of clear removable aligners for orthodontic purposes has received  
48 a great impulse (da CUNHA et al., n.d.; Tartaglia et al., 2021).

49 The first digitally designed and manufactured removable polyurethane aligners, based on the  
50 Invisalign<sup>TM</sup> system, were launched in 1998 by Align Technology (Santa Clara, CA, United States).  
51 Currently, clear aligners are produced all over the world by various companies (Galan-Lopez et al.,  
52 2019; Nemeč et al., 2020). Dedicated software are able to project and develop unique and  
53 personalized removable aligners, which perfectly fit with the patient's dentition, causing incremental  
54 tooth movements (Kravitz et al., 2009). Patients should wear each aligner for up to 22 hours per day  
55 for 7-14 days, according to the manufacturer's protocol (Al-Nadawi et al., 2021); the number of  
56 prescribed aligners depends on the amount of dental crowding and case complexity.

57 The thermoplastic materials used by aligner manufacturers mainly include polyethylene  
58 terephthalate (PET), polypropylene (PP), polycarbonate (PC), and polyurethanes (PU) (Daniele et al.,  
59 2020; Ho et al., 2021). These plastics can be prone to various environmental and mechanical factors  
60 which degrade them into smaller fragments, referred as secondary microplastics. In fact, the term  
61 “microplastic”, coined in 2004, is used to describe small plastic particles (Frias and Nash, 2019).

62 Most commonly, MPs are defined as synthetic polymer particles or fibers with a diameter of 1–5000  
63  $\mu\text{m}$  (Chain (CONTAM), 2016; Horton et al., 2017; Rocha-Santos and Duarte, 2015), even though the  
64 lower limit has been extended down to 100 nm by the European Food and Safety Authority (EFSA)  
65 (Chain (CONTAM), 2016). MPs can be distinguished into primary and secondary (Cole et al., 2011);  
66 the former are intentionally inserted in some products, such as toothpaste, face wash, cosmetics and  
67 industrial abrasives (da Costa et al., 2016), while the latter arise from the physical, chemical, and/or  
68 biological fragmentation of larger plastic objects during their use or when released in the environment  
69 (Cole et al., 2011). During the last decade, MPs emerged as “novel” pollutants and attracted  
70 considerable attention in the scientific community, due to their ubiquitous distribution and toxicity  
71 (Park and Park, 2021; Prata et al., 2020).

72 The ingestion of MPs by humans can be hazardous since some recent studies evidenced oxidative  
73 stress and inflammatory processes in animal models exposed to these microparticles (Yang et al.,  
74 2022). Moreover, the inability of the immune system to remove synthetic particles may lead to  
75 chronic inflammation and increase risk of neoplasia (Prata et al., 2020; Ragusa et al., 2021). In  
76 general, the potential toxicity of microparticles depends on their shape, chemical composition, and  
77 size (Triebkorn et al., 2019; Yang et al., 2022). Size is a crucial factor for the uptake, intended as the  
78 penetration into either cells or tissues beyond the epithelial surface (Triebkorn et al., 2019): it has  
79 been observed that very small particles are able to passively cross cell membranes, while larger ones  
80 require active endocytosis (Kettiger et al., 2013, p.). Generally, processes facilitating active uptake  
81 into tissues appear to work on particles up to 1  $\mu\text{m}$  (Zhu et al., 2013). As regards the shape, it  
82 influences the toxicity modifying interactions with cells and tissues: it has been demonstrated that  
83 microfibers interact with cells and tissues differently than microspheres, fragments, or films (Allegri  
84 et al., 2016).

85 Currently, optical and electronic microscopies, as well as spectroscopic techniques are widely  
86 employed to carry out a qualitative and quantitative characterization of MPs in different organic and  
87 biological matrices (Jenner et al., 2022; Kutralam-Muniasamy et al., 2023; Romano et al., 2022).

88 Scanning Electron Microscopy (SEM) represents an important tool for the quantification of MPs  
89 (Chen et al., 2020; Shi et al., 2022; Wang and Wang, 2018); moreover, thanks to its ability to create  
90 high- resolution images of the surfaces, it let obtain information on the micromorphology of  
91 microparticles, both in terms of size, shape and surface micromorphology and structure (Fries et al.,  
92 2013; Memè et al., 2022; Monterubbianesi et al., 2021; Tosco et al., 2021; Vitiello et al., 2022; Wang  
93 et al., 2017). Regarding the spectroscopic techniques, Raman Microspectroscopy (RMS) is a highly  
94 reliable technique for the detection and identification of MPs, since it allows to characterize not only  
95 the morphological features of microparticles but also their chemical composition in terms of both  
96 polymer matrices and pigments (Araujo et al., 2018; Di Renzo et al., 2021; Orilisi et al., 2021; Orsini  
97 et al., 2021; Ragusa et al., 2022). Furthermore, thanks to the high potential of light scattering, RMS  
98 offers the advantage of enabling the analysis of MPs as small as  $\sim 2 \mu\text{m}$  directly on filtration  
99 membranes (Jin et al., 2022; Ribeiro Claro et al., 2016).

100 In this *in vitro* study, for the first time, the potential detachment of microparticles by clear  
101 orthodontic aligners has been investigated. To this aim, orthodontic clear aligners provided by seven  
102 different manufacturers were submitted to a 7-days protocol in artificial saliva to simulate the  
103 mechanical friction generated by teeth. The detached MPs were then analyzed by Raman  
104 Microspectroscopy and Scanning Electron Microscopy. This is an important and actual topic, since  
105 clear orthodontic aligners are widely used every day all over the world. The detached small polymer  
106 fragments can be considered as secondary MPs and their ingestion could cause oxidative and  
107 inflammatory processes in orthodontic patients (Galloway, 2015).

108

## 109 **2. Materials and methods**

### 110 *2.1 Materials*

111 The orthodontic clear aligners, derived from the same STL file, were provided by the following  
112 manufacturers: Alleo (**AL**, Digital Service Leone s.r.l, Florence, Italy); Flexi Ligner (**FL**, Roma,  
113 Italy); F22 Aligner (**F22**, Sweden & Martina Spa, Padova, Italy); Invisalign (**INV**, Align Technology,

114 Mexico); Lineo (**LIN**, Micerium Lab, Milan, Italy); ArcAngel (**ARC**, Network Gruppo Dextra,  
115 Modena, Italy), and Ortobel Aligner (**OR**, Bergamo, Italy).

116 The artificial saliva was prepared by Biotène Oral balance (GSK, England), and was composed by  
117 purified water, hydrogenated hydrolyzed starch, xylitol, hydroxyethylcellulose, polymethacrylate,  
118 beta-d-glucose, lactoperoxidase (12,000 units), lysozyme (12mg), lactoferrin (12mg), glucose  
119 oxidase (12,000 units), potassium thiocyanate, aloe vera, without any treatment.

120

## 121 *2.2 Samples' treatment*

122 A specific protocol was set to simulate the oral cavity conditions, in which patients simultaneously  
123 wear two aligners for 7 days, one for each dental arch (Al-Nadawi et al., 2021). To this purpose, two  
124 samples of each manufacturer were immersed in 50 ml of artificial saliva in a glass beaker for 7 days.  
125 The beaker was covered with an aluminum foil throughout the experiment and positioned onto a  
126 magnetic hot plate (SuperNuova+™ Stirrer series, Thermo Scientific™, Loughborough, UK) at a  
127 constant temperature of 37° C. A cylinder-shape magnetic stirring bar (6 × 25 mm) coated with Teflon  
128 was added to create a rotating magnetic field. Each group of samples was stirred for 5 hours/day, in  
129 order to simulate the patient physiological teeth friction. In particular, during spontaneous swallowing  
130 the dental arches, and as a consequence the clear aligners, come into contact, creating a mechanical  
131 friction. For this reason, based on the spontaneous swallow frequency reported in literature (0.98/min)  
132 (Bulmer et al., 2021), and considering that in general, to ensure the best effectiveness, aligners must  
133 be worn for 20/22 hours/day (Hartshorne and Wertheimer, 2022), the number of spontaneous  
134 swallowing is around 1235. The cylinder-shape magnetic stirring bar used has been calibrated to  
135 achieve 250 rotations/hour. Thus, we performed 1250 rotations in 5 hours/day. After 7 days, the  
136 artificial saliva was filtered through 1.6 µm pore-size filter membranes (Whatman GF/A), with a  
137 diameter of 47 mm, by a vacuum pump connected to a filter tunnel. Filter membranes were dried at  
138 room temperature and stored in glass Petri dishes until Raman Microspectroscopy (RMS) and  
139 Scanning Electron Microscopy (SEM) analyses. The experiment was performed in triplicate.

140

### 141 *2.3 Raman Microspectroscopy analysis*

142 RMS analysis was carried out at the ARI Laboratory (Department of Life and Environmental  
143 Sciences, Polytechnic University of Marche, Ancona, Italy) by using a XploRA Nano Raman  
144 Microspectrometer (Horiba Scientific). All the filter membranes, including those deriving from the  
145 procedural blanks, were inspected by visible light using a  $\times 10$  objective (Olympus MPLAN10 $\times$ /0.25).  
146 The detected MPs were morphologically characterized by a  $\times 100$  objective (Olympus  
147 MPLAN100 $\times$ /0.90) and then directly analyzed on the filter by RMS (spectral range 200–1800  $\text{cm}^{-1}$ ,  
148 532 nm or 785 nm laser diode, 600 lines per mm grating). Spectra were dispersed onto a 16-bit  
149 dynamic range Peltier-cooled CCD detector; the spectrometer was calibrated to the 520.7  $\text{cm}^{-1}$  line  
150 of silicon prior to spectral acquisition. To reduce noise and enhance spectrum quality, raw Raman  
151 spectra were subjected to polynomial baseline correction and vector normalization (Labspec 6  
152 software, Horiba Scientific). The polymer matrix of the detected particles was identified by  
153 comparing the collected Raman spectra with spectral libraries of polymers obtained by measuring  
154 standard polymers/compounds (KnowItAll software, John Wiley & Sons, Inc., Hoboken, NJ, USA)  
155 (Chen et al., 2020; Fries et al., 2013). Similarities of more than 80 of the Hit Quality Index (HQI)  
156 were considered satisfactory.

157

### 158 *2.4 Scanning Electron Microscopy analysis*

159 SEM analysis was performed at the Centre for Electron Microscopy – CISMIN (Department of  
160 Materials, Environmental Science and Urban Planning, Polytechnic University of Marche, Ancona,  
161 Italy). From the same filters analyzed by RMS, supposing a homogeneous distribution of fragments,  
162 a representative circular portion with a diameter of *ca.* 20 mm was cropped (Hidalgo-Ruz et al., 2012);  
163 more in detail, the original filter had an area of *ca.* 1734.1  $\text{mm}^2$ , while the cropped filter of *ca.* 314.0  
164  $\text{mm}^2$ . The cropped filters were mounted on aluminum stubs, sputter-coated with gold and observed

165 by a TESCAN VEGA 3 LMU scanning electron microscope. SEM operated at 10 kV and at variable  
166 working distance with secondary electron detector (SE).

167 SEM images were acquired at different magnifications to investigate the MPs number,  
168 morphology, and size. In particular, the MPs count was performed through the visual inspection  
169 (Wang and Wang, 2018); to improve count accuracy and reduce the subjectivity of the examiner, the  
170 analyses were performed according to the following criteria: (i) the entire area of the cropped filter  
171 was inspected, starting from the upper left to the lower right; (ii) aggregated MPs were considered  
172 only one time; (iii) suspected particles were excluded (Chen et al., 2020; Song et al., 2015; Wang and  
173 Wang, 2018). The morphology and the size of all the detected MPs were also obtained.

174

## 175 *2.5 Quality Assurance and Control*

176 A plastic-free protocol was adopted to avoid microplastic contamination. Cotton laboratory coats  
177 and single-use latex gloves were worn during all phases of the experiment. The phases of mechanical  
178 friction and filtration were carried out in a dedicated room. Routinely employed plastic tools were  
179 replaced with glass ones, and were washed using dishwashing liquid, triple rinsed with 70% ethanol,  
180 and finally rinsed with 1.6  $\mu\text{m}$  filtered deionized water. Work surfaces were thoroughly washed with  
181 70% ethanol prior to starting all procedures and during the experimental time.

182 Moreover, environmental and procedural blanks were prepared and thoroughly analyzed to detect  
183 microplastic contamination deriving from the laboratory environment and from other external  
184 sources. As regards environmental blanks, a filter membrane soaked with 1.6  $\mu\text{m}$  filtered deionized  
185 water was placed into an uncovered Petri dish and positioned each day in the above-mentioned  
186 dedicated room. The filters deriving from environmental and procedural blanks were first inspected  
187 by stereomicroscope.

188

## 189 *2.6 Statistical Analysis*

190 Normally distributed data of particles' size were presented as mean  $\pm$  S.D. Significant differences  
 191 between experimental groups were determined by means of a factorial analysis of variance (one-way  
 192 ANOVA), followed by Tukey's multiple comparisons test, by the statistical software Prism6  
 193 (Graphpad Software, Inc. USA). One-way ANOVA was used to compare the means of **AL**, **F22**, **FL**,  
 194 **LIN**, **OR**, **ARC**, and **INV** groups to make inferences about the population means. Statistical  
 195 significance was set at  $p < 0.05$ .

196

### 197 3. Results

198 Filters from all the experimental groups were first analyzed by RMS; then, they were cut, and the  
 199 cropped circular portions were submitted to SEM evaluation. The details, including the chemical  
 200 composition, number, and size, of all the microparticles detected in the three replicates, are reported  
 201 in Table 1. As regards the number of MPs, it represents the number of microparticles found in the cut  
 202 filter portions (diameter *ca.* 20 mm). It is noteworthy that both in environmental and procedural  
 203 blanks, no microparticles of PET and PU were found.

**Table 1**

Manufacturer, polymer matrix, number, and mean size of the MPs detected in the three replicates of the following aligners: Alleo (**AL**), Flexi Ligner (**FL**), Lineo (**LIN**), ArcAngel (**ARC**), and Ortobel (**OR**); (B) F22 Aligner (**F22**), and Invisalign (**INV**).

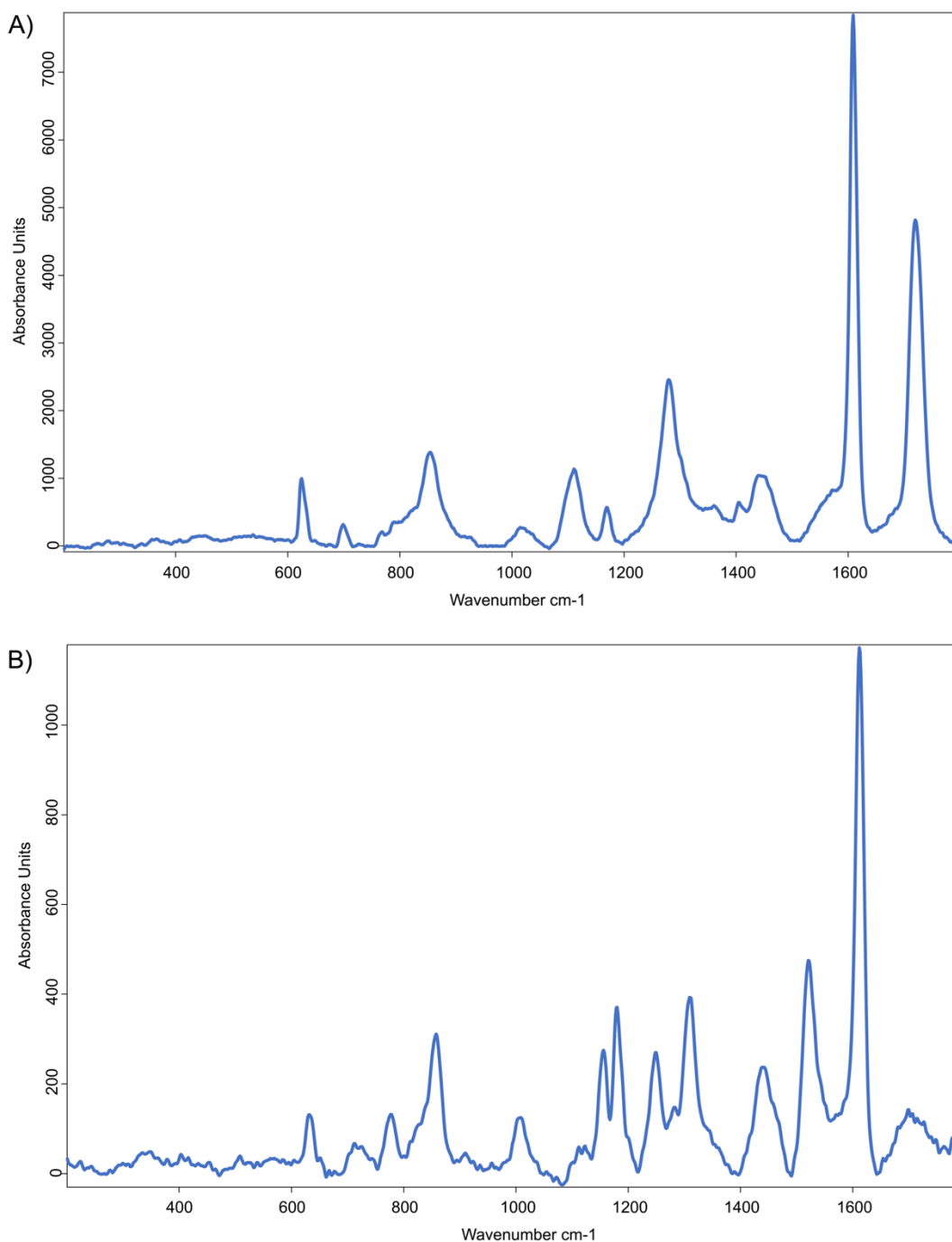
Manufacturer	Polymer*	Replicate (#)	N. of MPs	Mean size ( $\mu\text{m}$ )	Smallest ( $\mu\text{m}$ )	Largest ( $\mu\text{m}$ )
<b>AL</b>	PET	#1	12	13.72 $\pm$ 7.07	3.76	22.11
		#2	14	16.16 $\pm$ 8.67	3.41	28.31
		#3	12	16.09 $\pm$ 7.25	4.78	28.9
<b>FL</b>	PET	#1	10	20.37 $\pm$ 7.23	7.77	36.39
		#2	8	20.12 $\pm$ 10.74	9.18	28.36
		#3	12	19.29 $\pm$ 11.76	8.34	45.70
<b>F22</b>	PU	#1	11	16.12 $\pm$ 9.41	4.55	31.58
		#2	10	19.49 $\pm$ 12.69	4.83	38.28
		#3	12	18.80 $\pm$ 9.92	8.97	34.91
<b>INV</b>	PU	#1	7	16.64 $\pm$ 9.66	3.13	31.97
		#2	5	12.12 $\pm$ 5.70	3.96	18.57
		#3	7	15.81 $\pm$ 11.37	3.85	34.20

<b>LIN</b>	PET	#1	13	21.02 ± 12.15	5.89	55.8
		#2	14	22.71 ± 8.87	6.92	41.07
		#3	17	20.13 ± 8.43	7.10	37.70
<b>ARC</b>	PET	#1	17	25.09 ± 20.95	7.47	94.49
		#2	20	23.19 ± 11.35	5.62	44.80
		#3	16	24.57 ± 11.37	9.10	42.30
<b>OR</b>	PET	#1	14	20.74 ± 13.04	9.16	61.0
		#2	15	23.19 ± 11.35	7.40	43.81
		#3	18	24.57 ± 10.65	9.10	42.3

\* PET: polyethylene terephthalate; PU: polyurethane. N. of MPS: number of MPs counted in the cropped filter with a diameter of *ca.* 20 mm.

204

205 As regards the chemical composition, the representative RMS spectra of all the MPs confirmed  
 206 the presence of two different polymers: polyethylene terephthalate (PET) for **AL**, **FL**, **LIN**, **ARC**,  
 207 and **OR** samples, and polyurethane (PU) for **F22** and **INV** (Fig. 1).

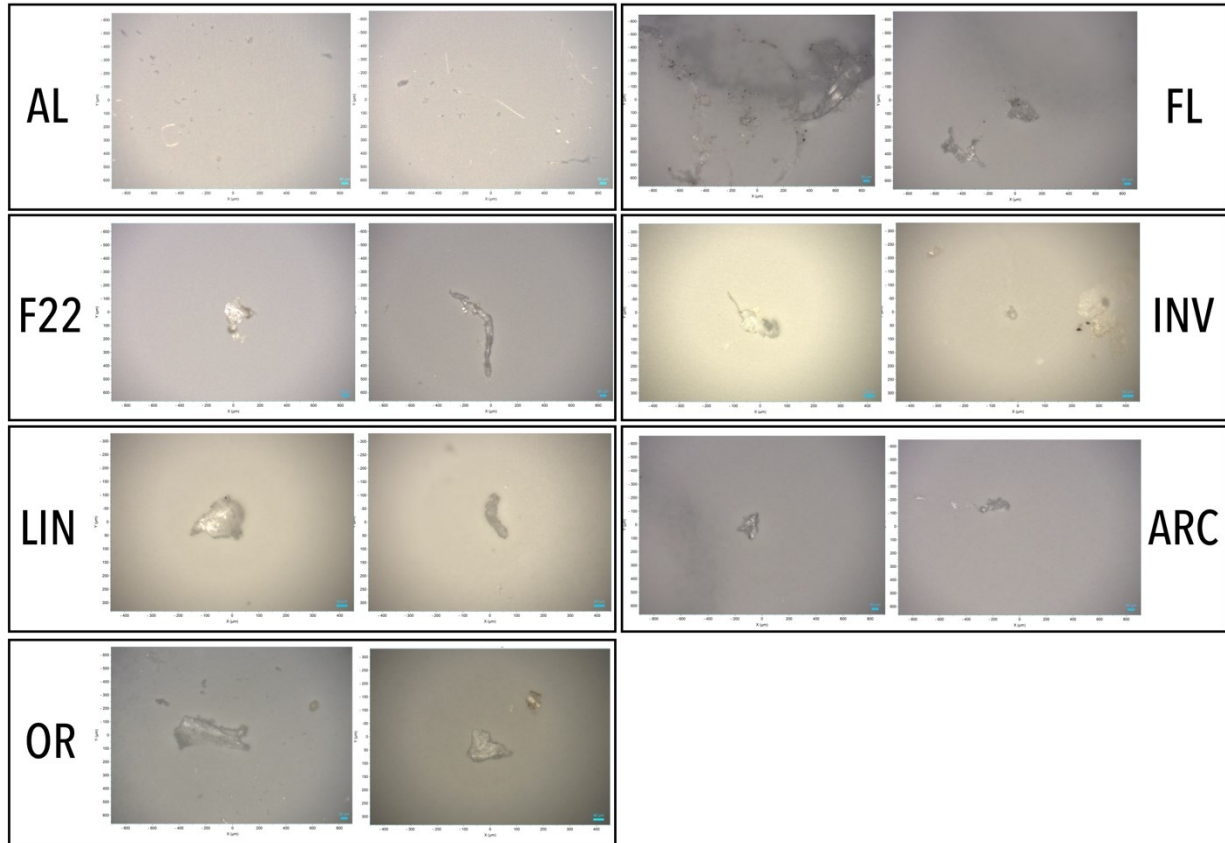


**Fig 1.** Representative RMS spectra collected on the microparticles detached from the aligners. Spectra (A) and (B) were ascribable respectively to polyethylene terephthalate and polyurethane. Spectrum (A): Alleo (**AL**), Flexi Ligner (**FL**), Lineo (**LIN**), ArcAngel (**ARC**), and Ortobel (**OR**). Spectrum (B) F22 Aligner (**F22**), and Invisalign (**INV**).

208

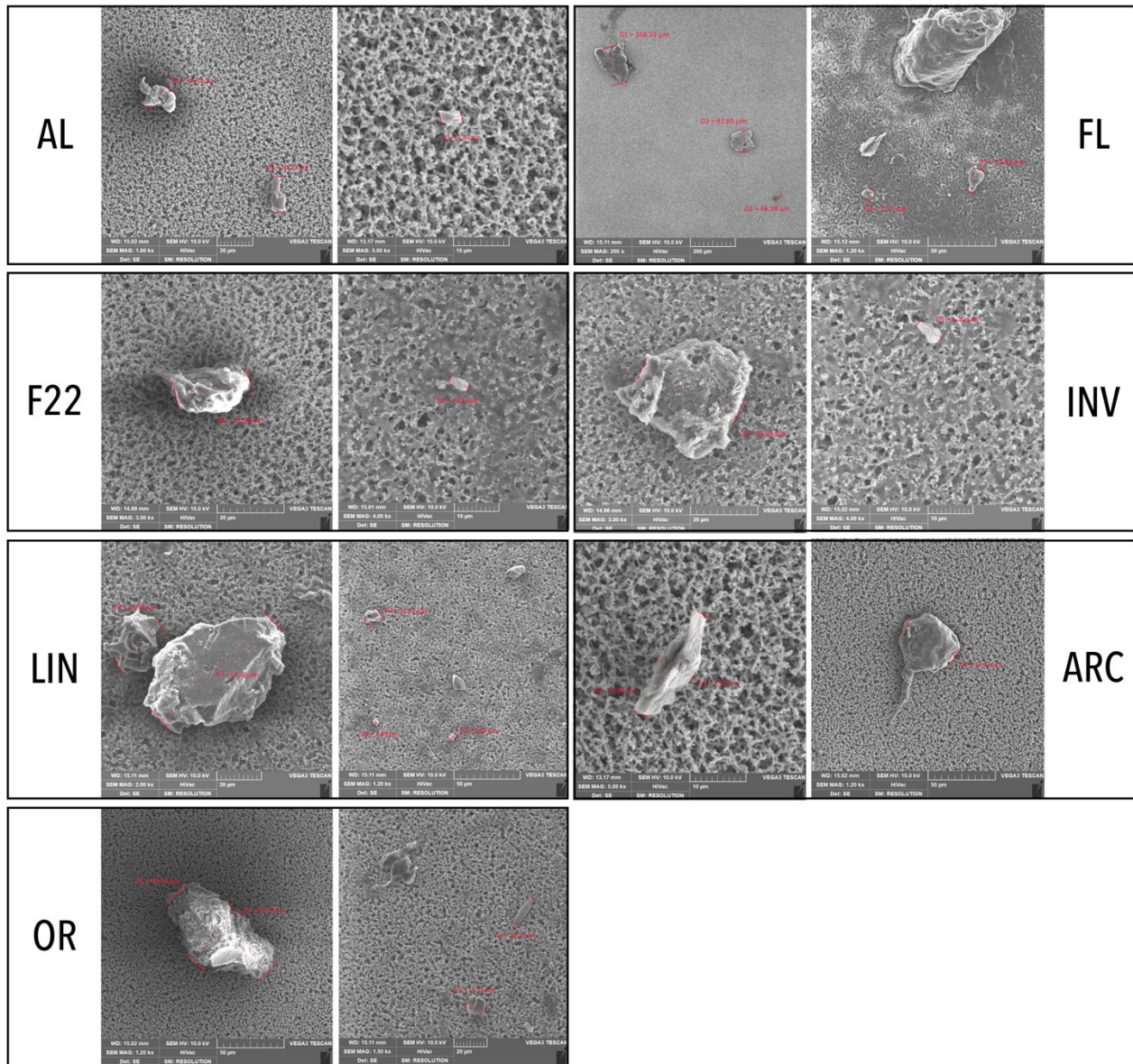
209 In Fig. 2, the microphotographs collected with the light microscope (100x magnification) of some  
 210 representative MPs found in each group were reported. In almost all cases, the detached  
 211 microparticles appeared as irregular fragments with different shape and size: more in detail, an almost  
 212 spherical shape was observed in **AL**, **FL**, **INV**, and **OR** groups, while a fiber shape was identified in

213 **F22, LIN** and **ARC** ones. Moreover, in **FL, INV** and **ARC**, some of the identified MPs were  
214 pigmented with blue and black colors due the writings on the aligners.  
215



**Fig. 2.** Microphotographs of some selected microparticles detached from the following aligners: **AL:** Alleo; **FL:** Flexi Ligner; **F22:** F22 Aligner; **INV:** Invisalign; **LIN:** Lineo; **ARC:** ArcAngel; **OR:** Ortobel Aligner (100x magnification, Olympus MPLAN100×/0.90)

216  
217 In Fig. 3, SEM micrographs of representative MPs collected for each group are shown.  
218 Interestingly, the high magnification (2000x – 3000x) revealed that MPs deriving from **F22** and **INV**  
219 groups, appeared as an aggregate of microspheres, while those detected in all the other groups seemed  
220 to have a more homogeneous surface.  
221



**Fig. 3.** Scanning electron micrographs collected at different magnifications on some selected MPs detached from the following aligners: **AL**: Alleo; **FL**: Flexi Ligner; **F22**: F22 Aligner; **INV**: Invisalign; **LIN**: Lineo; **ARC**: ArcAngel; **OR**: Ortobel. For each micrograph, the sizes of MPs were reported ( $\mu\text{m}$ ).

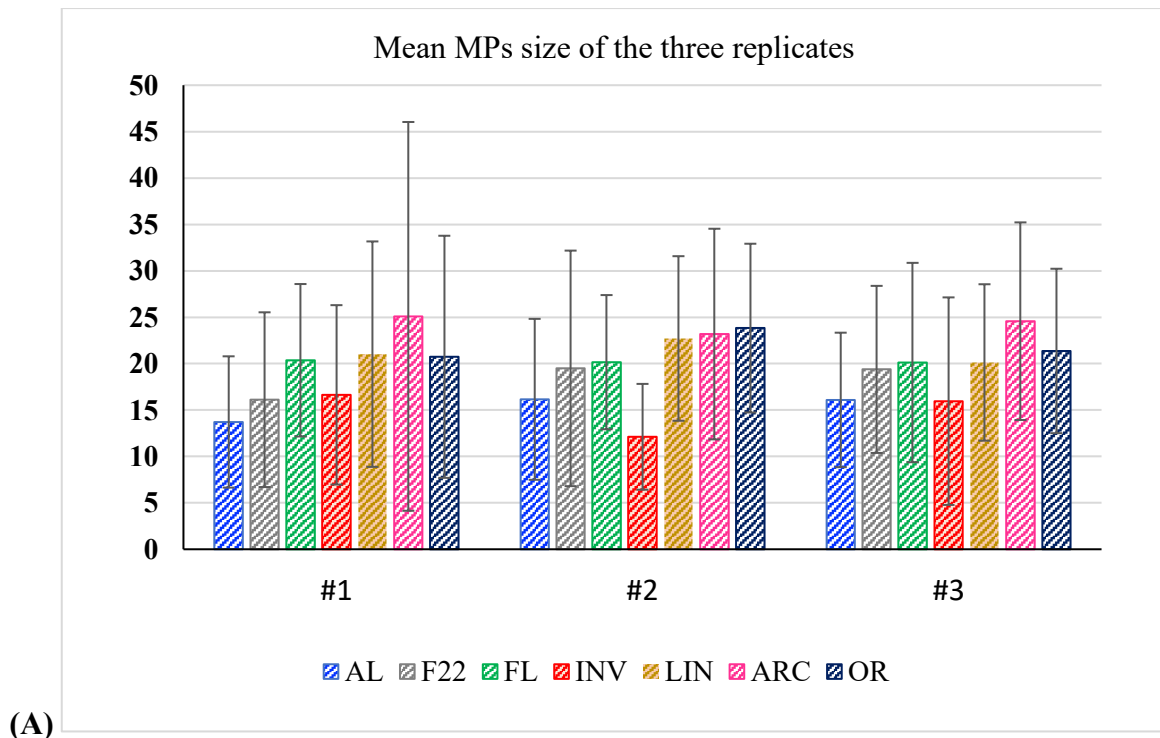
222

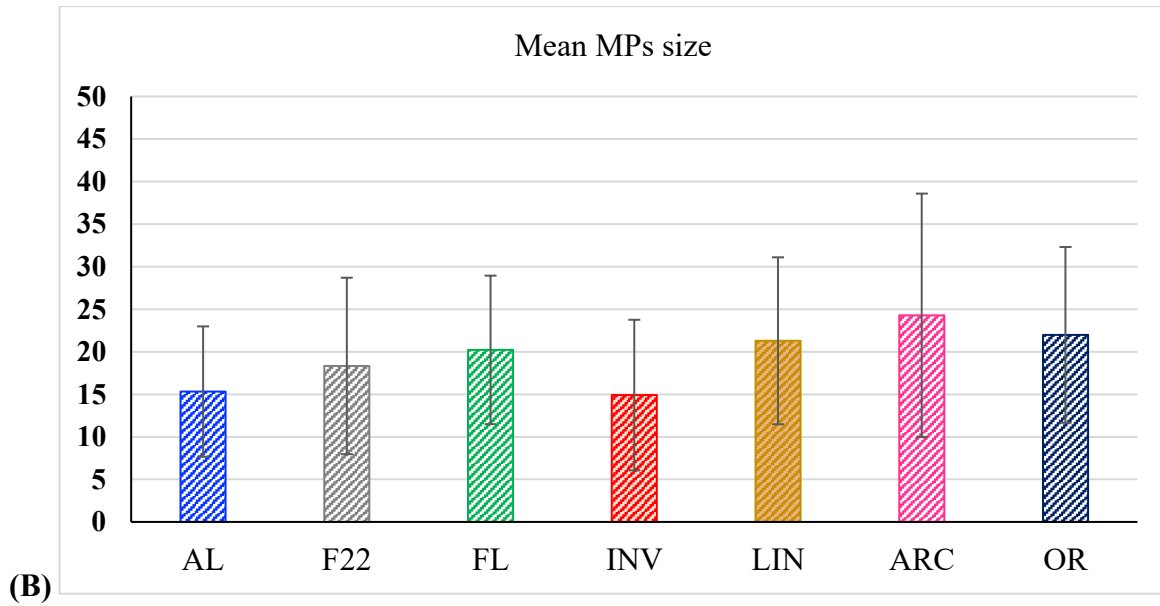
223 The average number of MPs, found in the different aligners and derived from the three replicates,  
 224 revealed statistically significant differences between the tested groups ( $p < 0.05$ ). In particular, more  
 225 than 10 MPs were counted in **AL** ( $13 \pm 1$ ), **F22** ( $11 \pm 1$ ), **FL** ( $10 \pm 2$ ), **LIN** ( $15 \pm 2$ ), **ARC** ( $18 \pm 2$ )  
 226 and **OR** ( $16 \pm 2$ ) ( $p > 0.05$ ), while only in **INV**, the number of MPs was lower than 10 ( $6 \pm 1$ ) ( $p < 0.05$ ).  
 227 Relating these data to the entire filter, with a diameter of 47 mm, the following values of counted  
 228 MPs were found: N.  $72 \pm 6$  in **AL**; N.  $61 \pm 6$  in **F22**; N.  $55 \pm 11$  in **FL**; N.  $83 \pm 11$  in **LIN**; N.  $88 \pm$

229 11 in **OR**; N.  $99 \pm 11$  in **ARC**; N.  $33 \pm 6$  in **INV**. Hence, the highest number was found in **ARC** and  
230 the lowest one in **INV**.

231 As regards MPs' size in the three replicates, no statistically significant differences were  
232 observed ( $p > 0.05$ ) (Fig. 4A). However, considering the average size of the three replicates (Fig. 4B),  
233 the lowest ones were found both in **INV** ( $14.91 \pm 8.85 \mu\text{m}$ ), with the smallest MP detected of  $3.13 \mu\text{m}$ ,  
234 and in **AL** ( $15.32 \pm 7.66 \mu\text{m}$ ), with the smallest MP detected of  $3.41 \mu\text{m}$ . In **F22**, an average size of  
235  $18.33 \pm 10.37 \mu\text{m}$  was found, with the lowest MPs' size of  $4.55 \mu\text{m}$ . **FL**, **LIN**, **ARC** and **OR** groups  
236 presented MPs in the range of  $20\text{-}30 \mu\text{m}$  ( $20.22 \pm 8.73$ ,  $21.29 \pm 9.81$ ,  $24.28 \pm 14.31$  and  $21.98 \pm 10.33$ ,  
237 respectively). In Fig. 5, the distribution in percentage of the MPs sizes, subdivided into 3 ranges ( $< 5 \mu\text{m}$ ,  
238  $5\text{-}20 \mu\text{m}$  and  $> 20 \mu\text{m}$ ) for each group is also reported. Furthermore, univariate Chi square test  
239 revealed a highly significant association ( $p < 0.0001$ ) between the distribution of particles' size and  
240 the different typologies of aligners.

241

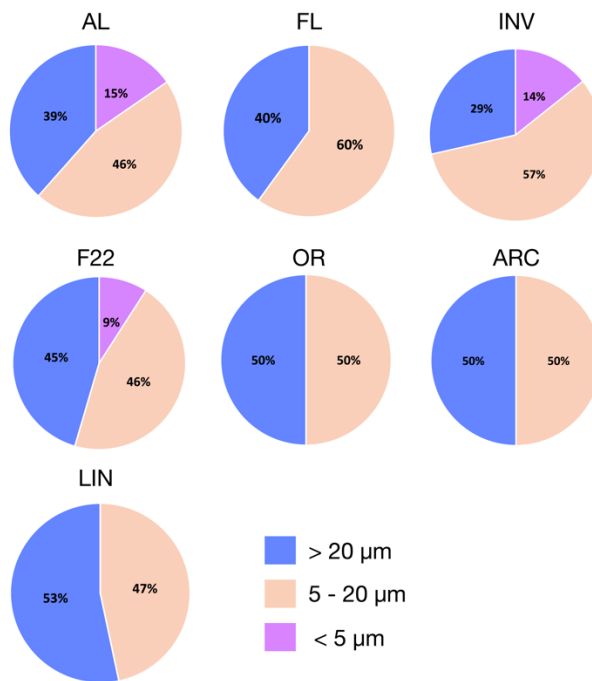




**Fig. 4.** (A) Mean size ( $\mu\text{m}$ ) and standard deviation of MPs detected in the experimental groups, subdivided in the three replicates (#1, #2, #3); (B) mean size ( $\mu\text{m}$ ) and standard deviation of MPs of the three replicates. **AL:** Alleo; **F22:** F22 Aligner; **FL:** Flexi Ligner; **INV:** Invisalign; **LIN:** Lineo; **ARC:** ArcAngel; **OR:** Ortobel Aligner

242

243



**Fig. 5.** Distribution in percentage (%) of the mean MPs sizes of the three replicates, subdivided into 3 ranges ( $< 5 \mu\text{m}$ ,  $5-20 \mu\text{m}$  and  $> 20 \mu\text{m}$ ), for each group: **AL** (Alleo); **FL** (Flexi Ligner); **F22** (F22 Aligner); **INV** (Invisalign); **LIN** (Lineo); **ARC** (ArcAngel); **OR** (Ortobel Aligner).

244

#### 245 4. Discussion

246 Since the introduction of clear aligners with the Invisalign™ brand, distributed by the US company  
247 Align Technology® (Kuo and Miller, 2003; Meier et al., 2003), during the years, the commercial offer  
248 has been significantly enriched with national and international competitor brands. Nowadays, clear  
249 aligners, with the widespread popularity due to their better comfort and aesthetics, are an integral part  
250 of orthodontic treatments and are receiving increased attention as an alternative to conventional  
251 braces, in both young and adult patients (Pacheco-Pereira et al., 2018; Weir, 2017). Thermoplastic  
252 polymers are the most common materials of which aligners are made (Condo' et al., 2018).  
253 Biomechanical properties play a key role in the performance and in obtaining the desired orthodontic  
254 tooth movement (Kohda et al., 2013). The most used materials are polyurethane, polyester, and  
255 polyethylene terephthalate. Many spectrophotometric studies have already analyzed the composition  
256 of clear aligners to confirm the chemical structure, stated by the manufacturers (Tamburrino et al.,  
257 2020).

258 In the last years, many efforts have been made to provide clinical guidelines for optimal aligner  
259 wear protocols (Al-Nadawi et al., 2021; Bilello et al., 2022; Hartshorne and Wertheimer, 2022;  
260 Putrino et al., 2021; Robertson et al., 2020): in general, to ensure the best effectiveness, aligners must  
261 be worn for 20/22 hours/day, and they should be changed every 14-days (Hartshorne and Wertheimer,  
262 2022). Recently, this prescription has been questioned (Bilello et al., 2022). In fact, Al-Nadawi et al.  
263 suggested that a 7-day protocol can be generally sufficient since there was no significant clinical  
264 difference compared with a 10-day or a 14-day protocol (Al-Nadawi et al., 2021).

265 However, the daily wearing of aligners by patients inevitably lead to a continuous frictional contact  
266 between the occlusal aligner surfaces, and this mechanism could allow a possible detach of plastic  
267 fragments from the thermoplastic material in the oral cavity. This fact, coupled with the large number  
268 of hours per day and the long period aligners are recommended to be worn for achieving the desired  
269 positive results, generates a growing concern about the risks associated with the exposure and intake  
270 of microplastics in orthodontic patients.

271 Until today, several studies have been performed to evaluate the stability of these thermoplastic  
272 materials in terms of mechanical properties, aging, colorimetric alteration after exposure to highly  
273 pigmented foods, and chemical changes during wearing time which could compromise the force  
274 delivery capacity and treatment efficacy (Bernard et al., 2020; Liu et al., 2016; Lombardo et al.,  
275 2017b; Papadopoulou et al., 2019). Hence, this is the first *in vitro* study which demonstrates that clear  
276 aligners produced from different manufacturers and subjected for 7 days to artificial mechanical  
277 friction, can release microparticles with variable shapes and sizes. MPs were chemically characterized  
278 by RMS and evaluated in terms of shape and sizes, using optical and scanning electron microscopies.

279 A specific protocol, based on the mean wearing time that emerged from the scientific literature,  
280 was set up to reproduce the mechanical friction to which aligners are subjected into the oral cavity  
281 (Al-Nadawi et al., 2021). In this regard, in all the tested groups, the mechanical friction led to the  
282 detachment of MPs with irregular profiles and with sizes ranging from 3  $\mu\text{m}$  to 50  $\mu\text{m}$ . All the detected  
283 MPs resulted made by two type of thermoplastic polymers: polyethylene terephthalate (in the case of  
284 **AL**, **FL**, **LIN**, **ARC**, and **OR** groups) and polyurethane (in the case of **F22** and **INV** groups) (Daniele  
285 et al., 2022; Ihssen et al., 2019; Lombardo et al., 2017a). As previously described, these microparticles  
286 can be classified as secondary microparticles, since they derive from the fragmentation of larger  
287 plastic items during their use (Cole et al., 2011).

288 Currently, there is growing scientific evidence about MPs in humans, with an estimated total intake  
289 of 39-52 thousand MPs per person per year, mainly through ingestion (Cox et al., 2019; Prata, 2018;  
290 Prata et al., 2020). According to the scientific literature, the primary health effects of ingested MPs  
291 are triggered from the digestive system, causing direct damage not only at local level, such as irritation  
292 or intestinal dysbiosis, but also at systemic level (Tamargo et al., 2022; Yee et al., 2021). To date, the  
293 changes of MPs during gastrointestinal digestion or colonic fermentation are scarcely explored.  
294 However, a recent study provided scientific evidence of modifications and potential effects of MPs  
295 during their passage through the digestive tract (Tamargo et al., 2022). Indeed, authors reported that  
296 PET MPs during gastrointestinal digestion showed structural changes, suggesting a potential

297 biodegradation probably driven by colonic microbiota, supporting the existence of an interaction  
298 between the colonic microbiota and PET MPs particles. Although there are few experimental  
299 researches on MPs metabolism in the human body, studies agree that their uptake is influenced by  
300 their shape and size (Triebkorn et al., 2019; Wieland et al., 2022). In particular, size represents a  
301 crucial factor, with larger particles requiring active endocytosis, while very small particles being able  
302 to passively cross membranes (Kettiger et al., 2013; Triebkorn et al., 2019; Yang et al., 2022).  
303 Indeed, particles > 20 µm are likely excreted from the gastrointestinal tract (Schwabl et al., 2019;  
304 Wieland et al., 2022). Conversely, microparticles ranging from 5 µm to 20 µm, at the gastrointestinal  
305 level, may pass through the epithelium by endocytosis mechanisms or by paracellular diffusion. After  
306 that, MPs are translocated by dendritic cells through the lymphatic circulation and reach the  
307 circulatory system (Prata et al., 2020). As regards our results, microparticles with a diameter of 5 -  
308 20 µm were found in all the attested aligners and represented the largest group, with a percentage  
309 higher than 50%, except that for **F22** and **LIN** (36% and 46%, respectively). A percentage range of  
310 30-50% was detected for MPs > 20 µm in all the aligners, while MPs < 5 µm were detected only in  
311 **AL** (17%), **F22** (18%) and **INV** (14%). In this context, it needs to be considered if these MPs could  
312 pass the gastrointestinal tract through para and/or transcellular manner. Florence et al. reported that  
313 the uptake of MPs by M-cells into the Peyer's patches (lymphoid follicles in the small intestine) plays  
314 a significant role (Florence, 1997). Indeed, the presence of MPs could cause aggregations of  
315 macrophages, granulation tissue and foreign body response, with inflammation and oxidative stress  
316 (Paul et al., 2020; Urban et al., 2000; Willert and Semlitsch, 1996). In this light, it is suggested to use  
317 this type of orthodontic treatment with caution in growing children.

318 Recent studies showed that the leaching of monomers from MPs could contribute to their toxicity  
319 (Mastrangelo et al., 2002; Xu et al., 2003). MPs deriving from **F22** and **INV** aligners appeared as  
320 aggregates of microspheres, and, hence, they could lead to a further detachment of microparticles  
321 with smaller diameter and with higher toxicity into the gastrointestinal tract. Moreover, in **FL**, **INV**  
322 and **ARC** aligners, some of the identified MPs appeared blue or black pigmented. This finding,

323 probably related to the ink used to identify the aligner, could be explained by the fact that these areas  
324 may be less resistant to mechanical friction, leading to easier detachment of the MPs.

325 Another important factor, which could lead to a different level of MPs detachment, could be the  
326 processing in the manufacturing techniques (Alhendi et al., 2022; Eliades et al., 1999). Clear aligners,  
327 indeed, can be thermoformed on the serial digital 3D models, considering the conventional  
328 fabrication, or can be direct 3D printed, representing the new approach (Maspero and Tartaglia,  
329 2020). This technology allows to manufacture components layer-by-layer (such as stereolithography,  
330 selective laser sintering and fused deposition modelling), instead of common manufacturing methods  
331 that rely on molding, machining or other subtractive methods (Athirasala et al., 2018). From the  
332 analyzed groups, only Invisalign<sup>®</sup> aligners are 3D printed, based on the application of the  
333 stereolithography technology (Tartaglia et al., 2021). In the other groups, clear aligners are produced  
334 using the thermoformed method. According to our results, a highly significant association ( $p <$   
335  $0.0001$ ) between the distribution of particles' size and the different typologies of aligners emerged.  
336 Indeed, in INV aligners the detachment of MPs appeared the lowest in number respect to the other  
337 groups. This finding could be due associated to the thermoforming process which could significantly  
338 change the material properties in response to the heat generation used to form the material around the  
339 3D model. In this light, our results agree with the scientific literature. Studies showed that  
340 thermoplastic-made aligners are reactive during their use to the intraoral environment, such as body  
341 temperature, humidity of oral cavity and salivary enzymes, which may intrinsically affect the aligner  
342 and modify its original size and mechanical properties (Martina et al., 2019; Ryokawa et al., 2006).  
343 Thus, the alterations produced by the thermoforming process and the intraoral environment on the  
344 aligner structure, probably caused an alteration of the mechanical properties, with the consequent  
345 detachment of MPs. Furthermore, the thermoformed materials showed more cytotoxicity respect to  
346 directly 3D printed clear aligners, most likely due to the release of monomers in relation to the  
347 increasing temperature in the thermoplastic process (Martina et al., 2019). Conversely, studies on the

348 cytotoxicity of directly 3D printed clear aligners from three different materials, concluding that  
349 Invisalign<sup>®</sup> material represented the least cytotoxic (Tartaglia et al., 2021).

350 A limitation of this study could be ascribed to the difficulty of *in vitro* replicating the mechanical  
351 friction that occurs between the dental arches throughout the daily wearing. However, since in the  
352 oral cavity other factors could also contribute in the deterioration of the orthodontic clear aligners,  
353 we are confident that our findings underestimate the MPs detachment (Fang et al., 2020). Since there  
354 are few experimental studies on microplastic metabolism in the human body, it is judged that caution  
355 is needed in the interpretation of the present results. Furthermore, since the orthodontic treatment  
356 occurs in a short period of time, around 16.9±5.7 months (Borda et al., 2020), depending on the  
357 severity of the malocclusion and on the compliance of the patient (Torsello et al., 2022), the  
358 detachment of MPs and the consequent ingestion take place in a limited period. Thus, clear aligners,  
359 which represent a well-tolerated removable appliance, could be safely used. Nevertheless, future  
360 studies are needed to evaluate MPs detachments at different wearing time.

361

## 362 **5. Conclusions**

363 This *in vitro* study highlighted, for the first time, the detachment of MPs from commercial clear  
364 aligners, used for orthodontic treatments, due to their mechanical friction. This evidence could  
365 represent a great concern since it could impact the human general health. However, it is important to  
366 point out that in all groups, most of MPs had dimensions greater than or equal to 20 µm, and hence,  
367 they could be likely excreted from the gastrointestinal tract. As regards MPs with a smaller size (lower  
368 than 5 µm), which could be able to cross membranes and gut epithelium' barrier, this component  
369 represents only a small percentage. Therefore, the use of clear aligners limited for a short period of  
370 time can be considered a safe and valid orthodontic treatment. However, it is still mandatory to  
371 increase efforts in the scientific research to identify and test new materials for clear aligners and the  
372 wearing protocols.

373

374 **Author Contributions:** V.Q., Giu.O., F.V., Gio.O., G.M. conceptualization; Giu.O., F.V. data  
375 curation; Giu.O., V.N., F.V. formal analysis; V.N., F.V., Giu.O. funding acquisition; V.Q., Giu.O  
376 investigation; Giu.O., V.N. methodology; Gio.O., G.M. project administration; Gio.O., G.M, V.Q.  
377 resources; F.V., V.N. software; Gio.O., G.M. supervision; Gio.O., G.M. validation; Giu.O., V.N.,  
378 F.V. visualization; V.N., F.V., Giu.O roles/writing; V.Q., Giu.O, F.V., V.N., Gio.O., G.M. writing-  
379 review and editing.

380

381 **Funding:** The authors received no funding for this work.

## 382 **Acknowledgments**

383 The authors extend their gratitude to Adriano Di Cristoforo (Department of Materials, Environmental  
384 Science, and Urban Planning, Polytechnic University of Marche, Ancona, Italy) for his precious  
385 support in SEM measurements. The SISOPD (Società Italiana Stomatologia, Odontoiatria e Protesi  
386 Dentaria—Italian Society of Dentistry, Stomatology and Prosthodontics) Foundation is kindly  
387 acknowledged.

388

389 **Conflicts of Interest:** The authors declare that they have no known competing financial interest or  
390 personal relationships that could have appeared to influence the work reported in this study. Authors  
391 confirm that the submitted work, including images, is original and the journal policies have been  
392 reviewed. There are no conflicts of interest to disclose.

393

394 **Data Availability Statement:** The data presented in this study are available on request from the  
395 corresponding author.

396

## 397 **References**

- 398 Alhendi, A., Khounganian, R., Ali, R., Syed, S.A., Almudhi, A., 2022. Structural Conformation  
399 Comparison of Different Clear Aligner Systems: An In Vitro Study. *Dent J (Basel)* 10, 73.  
400 <https://doi.org/10.3390/dj10050073>
- 401 Allegri, M., Bianchi, M.G., Chiu, M., Varet, J., Costa, A.L., Ortelli, S., Blosi, M., Bussolati, O.,  
402 Poland, C.A., Bergamaschi, E., 2016. Shape-Related Toxicity of Titanium Dioxide Nanofibres.  
403 *PLoS One* 11, e0151365. <https://doi.org/10.1371/journal.pone.0151365>
- 404 Al-Nadawi, M., Kravitz, N.D., Hansa, I., Makki, L., Ferguson, D.J., Vaid, N.R., 2021. Effect of  
405 clear aligner wear protocol on the efficacy of tooth movement. *Angle Orthod* 91, 157–163.  
406 <https://doi.org/10.2319/071520-630.1>
- 407 Araujo, C.F., Nolasco, M.M., Ribeiro, A.M.P., Ribeiro-Claro, P.J.A., 2018. Identification of  
408 microplastics using Raman spectroscopy: Latest developments and future prospects. *Water Res* 142,  
409 426–440. <https://doi.org/10.1016/j.watres.2018.05.060>
- 410 Athirasala, A., Tahayeri, A., Thirivikraman, G., França, C.M., Monteiro, N., Tran, V., Ferracane, J.,  
411 Bertassoni, L.E., 2018. A dentin-derived hydrogel bioink for 3D bioprinting of cell laden scaffolds  
412 for regenerative dentistry. *Biofabrication* 10, 024101. <https://doi.org/10.1088/1758-5090/aa9b4e>
- 413 Bernard, G., Rompré, P., Tavares, J., Montpetit, A., 2020. Colorimetric and spectrophotometric  
414 measurements of orthodontic thermoplastic aligners exposed to various staining sources and  
415 cleaning methods. *Head & Face Medicine* 16. <https://doi.org/10.1186/s13005-020-00218-2>
- 416 Bilello, G., Fazio, M., Currò, G., Scardina, G.A., Pizzo, G., 2022. The Effects of Low-frequency  
417 Vibration on Aligner Treatment Duration: A Clinical Trial. *J Int Soc Prev Community Dent* 12,  
418 345–352. [https://doi.org/10.4103/jispcd.JISPCD\\_311\\_21](https://doi.org/10.4103/jispcd.JISPCD_311_21)
- 419 Borda, A.F., Garfinkle, J.S., Covell, D.A., Wang, M., Doyle, L., Sedgley, C.M., 2020. Outcome  
420 assessment of orthodontic clear aligner vs fixed appliance treatment in a teenage population with

421 mild malocclusions. *Angle Orthod* 90, 485–490. <https://doi.org/10.2319/122919-844.1>

422 Bulmer, J.M., Ewers, C., Drinnan, M.J., Ewan, V.C., 2021. Evaluation of Spontaneous Swallow  
423 Frequency in Healthy People and Those With, or at Risk of Developing, Dysphagia: A Review.  
424 *Gerontol Geriatr Med* 7, 23337214211041800. <https://doi.org/10.1177/23337214211041801>

425 Chain (CONTAM), E.P. on C. in the F., 2016. Presence of microplastics and nanoplastics in food,  
426 with particular focus on seafood. *EFSA Journal* 14, e04501.  
427 <https://doi.org/10.2903/j.efsa.2016.4501>

428 Chen, G., Fu, Z., Yang, H., Wang, J., 2020. An overview of analytical methods for detecting  
429 microplastics in the atmosphere. *TrAC Trends in Analytical Chemistry* 130, 115981.  
430 <https://doi.org/10.1016/j.trac.2020.115981>

431 Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the  
432 marine environment: A review. *Marine Pollution Bulletin* 62, 2588–2597.  
433 <https://doi.org/10.1016/j.marpolbul.2011.09.025>

434 Condo', R., Pazzini, L., Cerroni, L., Pasquantonio, G., Lagana', G., Pecora, A., Mussi, V., Rinaldi,  
435 A., Mecheri, B., Licoccia, S., Maiolo, L., 2018. Mechanical properties of “two generations” of teeth  
436 aligners: Change analysis during oral permanence. *Dent Mater J* 37, 835–842.  
437 <https://doi.org/10.4012/dmj.2017-323>

438 Cox, K.D., Covernton, G.A., Davies, H.L., Dower, J.F., Juanes, F., Dudas, S.E., 2019. Human  
439 Consumption of Microplastics. *Environ. Sci. Technol.* 53, 7068–7074.  
440 <https://doi.org/10.1021/acs.est.9b01517>

441 da Costa, J.P., Santos, P.S.M., Duarte, A.C., Rocha-Santos, T., 2016. (Nano)plastics in the  
442 environment – Sources, fates and effects. *Science of The Total Environment* 566–567, 15–26.  
443 <https://doi.org/10.1016/j.scitotenv.2016.05.041>

444 da CUNHA, T. de M.A., BARBOSA, I. da S., PALMA, K.K., n.d. Orthodontic digital workflow:  
445 devices and clinical applications. *Dental Press J Orthod* 26, e21spe6. [https://doi.org/10.1590/2177-](https://doi.org/10.1590/2177-6709.26.6.e21spe6)  
446 [6709.26.6.e21spe6](https://doi.org/10.1590/2177-6709.26.6.e21spe6)

447 Daniele, V., Macera, L., Taglieri, G., Di Giambattista, A., Spagnoli, G., Massaria, A., Messori, M.,  
448 Quagliarini, E., Chiappini, G., Campanella, V., Mummolo, S., Marchetti, E., Marzo, G., Quinzi, V.,  
449 2020. Thermoplastic Disks Used for Commercial Orthodontic Aligners: Complete Physicochemical  
450 and Mechanical Characterization. *Materials (Basel)* 13. <https://doi.org/10.3390/ma13102386>

451 Daniele, V., Macera, L., Taglieri, G., Spera, L., Marzo, G., Quinzi, V., 2022. Color Stability,  
452 Chemico-Physical and Optical Features of the Most Common PETG and PU Based Orthodontic  
453 Aligners for Clear Aligner Therapy. *Polymers* 14, 14. <https://doi.org/10.3390/polym14010014>

454 Di Renzo, L., Mascilongo, G., Berti, M., Bogdanović, T., Listeš, E., Brkljača, M., Notarstefano, V.,  
455 Gioacchini, G., Giorgini, E., Olivieri, V., Silvestri, C., Matiddi, M., D'Alterio, N., Ferri, N., Di  
456 Giacinto, F., 2021. Potential Impact of Microplastics and Additives on the Health Status of  
457 Loggerhead Turtles (*Caretta caretta*) Stranded Along the Central Adriatic Coast. *Water Air Soil*  
458 *Pollut* 232, 98. <https://doi.org/10.1007/s11270-021-04994-8>

459 Eliades, T., Eliades, G., Watts, D.C., 1999. Structural conformation of in vitro and in vivo aged  
460 orthodontic elastomeric modules. *Eur J Orthod* 21, 649–658. <https://doi.org/10.1093/ejo/21.6.649>

461 Fang, D., Li, F., Zhang, Y., Bai, Y., Wu, B.M., 2020. Changes in mechanical properties, surface  
462 morphology, structure, and composition of Invisalign material in the oral environment. *American*  
463 *Journal of Orthodontics and Dentofacial Orthopedics* 157, 745–753.  
464 <https://doi.org/10.1016/j.ajodo.2019.05.023>

465 Florence, A.T., 1997. The oral absorption of micro- and nanoparticulates: neither exceptional nor  
466 unusual. *Pharm Res* 14, 259–266. <https://doi.org/10.1023/a:1012029517394>

467 Frias, J.P.G.L., Nash, R., 2019. Microplastics: Finding a consensus on the definition. *Mar Pollut*  
468 *Bull* 138, 145–147. <https://doi.org/10.1016/j.marpolbul.2018.11.022>

469 Fries, E., Dekiff, J.H., Willmeyer, J., Nuelle, M.-T., Ebert, M., Remy, D., 2013. Identification of  
470 polymer types and additives in marine microplastic particles using pyrolysis-GC/MS and scanning  
471 electron microscopy. *Environ. Sci.: Processes Impacts* 15, 1949–1956.

472 <https://doi.org/10.1039/C3EM00214D>

473 Galan-Lopez, L., Barcia-Gonzalez, J., Plasencia, E., 2019. A systematic review of the accuracy and  
474 efficiency of dental movements with Invisalign®. *Korean J Orthod* 49, 140–149.  
475 <https://doi.org/10.4041/kjod.2019.49.3.140>

476 Galloway, T.S., 2015. Micro- and Nano-plastics and Human Health, in: Bergmann, M., Gutow, L.,  
477 Klages, M. (Eds.), *Marine Anthropogenic Litter*. Springer International Publishing, Cham, pp. 343–  
478 366. [https://doi.org/10.1007/978-3-319-16510-3\\_13](https://doi.org/10.1007/978-3-319-16510-3_13)

479 Hartshorne, J., Wertheimer, M.B., 2022. Emerging insights and new developments in clear aligner  
480 therapy: A review of the literature. *AJO-DO Clinical Companion* 2, 311–324.  
481 <https://doi.org/10.1016/j.xaor.2022.04.009>

482 Hidalgo-Ruz, V., Gutow, L., Thompson, R.C., Thiel, M., 2012. Microplastics in the Marine  
483 Environment: A Review of the Methods Used for Identification and Quantification. *Environ. Sci.*  
484 *Technol.* 46, 3060–3075. <https://doi.org/10.1021/es2031505>

485 Ho, C.-T., Huang, Y.-T., Chao, C.-W., Huang, T.-H., Kao, C.-T., 2021. Effects of different aligner  
486 materials and attachments on orthodontic behavior. *Journal of Dental Sciences* 16, 1001–1009.  
487 <https://doi.org/10.1016/j.jds.2021.01.011>

488 Horton, A.A., Walton, A., Spurgeon, D.J., Lahive, E., Svendsen, C., 2017. Microplastics in  
489 freshwater and terrestrial environments: Evaluating the current understanding to identify the  
490 knowledge gaps and future research priorities. *Sci Total Environ* 586, 127–141.  
491 <https://doi.org/10.1016/j.scitotenv.2017.01.190>

492 Ihssen, B.A., Willmann, J.H., Nimer, A., Drescher, D., 2019. Effect of in vitro aging by water  
493 immersion and thermocycling on the mechanical properties of PETG aligner material. *J Orofac*  
494 *Orthop* 80, 292–303. <https://doi.org/10.1007/s00056-019-00192-8>

495 Jenner, L.C., Rotchell, J.M., Bennett, R.T., Cowen, M., Tentzeris, V., Sadofsky, L.R., 2022.  
496 Detection of microplastics in human lung tissue using  $\mu$ FTIR spectroscopy. *Science of The Total*  
497 *Environment* 831, 154907. <https://doi.org/10.1016/j.scitotenv.2022.154907>

498 Jin, N., Song, Y., Ma, R., Li, J., Li, G., Zhang, D., 2022. Characterization and identification of  
499 microplastics using Raman spectroscopy coupled with multivariate analysis. *Analytica Chimica*  
500 *Acta* 1197, 339519. <https://doi.org/10.1016/j.aca.2022.339519>

501 Kesling, H.D., 1946. Coordinating the predetermined pattern and tooth positioner with conventional  
502 treatment. *Am J Orthod Oral Surg* 32, 285–293. [https://doi.org/10.1016/0096-6347\(46\)90053-1](https://doi.org/10.1016/0096-6347(46)90053-1)

503 Kettiger, H., Schipanski, A., Wick, P., Huwyler, J., 2013. Engineered nanomaterial uptake and  
504 tissue distribution: from cell to organism. *Int J Nanomedicine* 8, 3255–3269.  
505 <https://doi.org/10.2147/IJN.S49770>

506 Kohda, N., Iijima, M., Muguruma, T., Brantley, W.A., Ahluwalia, K.S., Mizoguchi, I., 2013.  
507 Effects of mechanical properties of thermoplastic materials on the initial force of thermoplastic  
508 appliances. *Angle Orthod* 83, 476–483. <https://doi.org/10.2319/052512-432.1>

509 Kravitz, N.D., Kusnoto, B., BeGole, E., Obrez, A., Agran, B., 2009. How well does Invisalign  
510 work? A prospective clinical study evaluating the efficacy of tooth movement with Invisalign. *Am J*  
511 *Orthod Dentofacial Orthop* 135, 27–35. <https://doi.org/10.1016/j.ajodo.2007.05.018>

512 Kuo, E., Miller, R.J., 2003. Automated custom-manufacturing technology in orthodontics. *Am J*  
513 *Orthod Dentofacial Orthop* 123, 578–581. <https://doi.org/10.1067/mod.2003.S0889540603000519>

514 Kutralam-Muniasamy, G., Shruti, V.C., Pérez-Guevara, F., Roy, P.D., 2023. Microplastic  
515 diagnostics in humans: “The 3Ps” Progress, problems, and prospects. *Science of The Total*  
516 *Environment* 856, 159164. <https://doi.org/10.1016/j.scitotenv.2022.159164>

517 Liu, C.-L., Sun, W.-T., Liao, W., Lu, W.-X., Li, Q.-W., Jeong, Y., Liu, J., Zhao, Z.-H., 2016.  
518 Colour stabilities of three types of orthodontic clear aligners exposed to staining agents. *Int J Oral*  
519 *Sci* 8, 246–253. <https://doi.org/10.1038/ijos.2016.25>

520 Lombardo, L., Martines, E., Mazzanti, V., Arreghini, A., Mollica, F., Siciliani, G., 2017a. Stress  
521 relaxation properties of four orthodontic aligner materials: A 24-hour in vitro study. *Angle Orthod*  
522 87, 11–18. <https://doi.org/10.2319/113015-813.1>

523 Lombardo, L., Martini, M., Cervinara, F., Spedicato, G.A., Oliverio, T., Siciliani, G., 2017b.  
524 Comparative SEM analysis of nine F22 aligner cleaning strategies. *Prog Orthod* 18, 26.  
525 <https://doi.org/10.1186/s40510-017-0178-9>

526 Macri, M., Murmura, G., Varvara, G., Traini, T., Festa, F., 2022. Clinical Performances and  
527 Biological Features of Clear Aligners Materials in Orthodontics. *Frontiers in Materials* 9.  
528 Martina, S., Rongo, R., Bucci, R., Razionale, A.V., Valletta, R., D'Antò, V., 2019. In vitro  
529 cytotoxicity of different thermoplastic materials for clear aligners. *Angle Orthod* 89, 942–945.  
530 <https://doi.org/10.2319/091718-674.1>

531 Maspero, C., Tartaglia, G.M., 2020. 3D Printing of Clear Orthodontic Aligners: Where We Are and  
532 Where We Are Going. *Materials (Basel)* 13, 5204. <https://doi.org/10.3390/ma13225204>

533 Mastrangelo, G., Fedeli, U., Fadda, E., Milan, G., Lange, J.H., 2002. Epidemiologic evidence of  
534 cancer risk in textile industry workers: a review and update. *Toxicol Ind Health* 18, 171–181.  
535 <https://doi.org/10.1191/0748233702th139rr>

536 Meier, B., Wiemer, K.B., Miethke, R.-R., 2003. Invisalign--patient profiling. Analysis of a  
537 prospective survey. *J Orofac Orthop* 64, 352–358. <https://doi.org/10.1007/s00056-003-0301-z>

538 Memè, L., Strappa, E.M., Monterubbianesi, R., Bambini, F., Mummolo, S., 2022. SEM and FT-  
539 MIR Analysis of Human Demineralized Dentin Matrix: An In Vitro Study. *Applied Sciences* 12,  
540 1480. <https://doi.org/10.3390/app12031480>

541 Monterubbianesi, R., Tosco, V., Bellezze, T., Giuliani, G., Özcan, M., Putignano, A., Orsini, G.,  
542 2021. A Comparative Evaluation of Nanohydroxyapatite-Enriched Hydrogen Peroxide Home  
543 Bleaching System on Color, Hardness and Microstructure of Dental Enamel. *Materials* 14, 3072.  
544 <https://doi.org/10.3390/ma14113072>

545 Nemeč, M., Bartholomaeus, H.M., H. Bertl, M., Behm, C., Ali Shokoohi-Tabrizi, H., Jonke, E.,  
546 Andrukhov, O., Rausch-Fan, X., 2020. Behaviour of Human Oral Epithelial Cells Grown on  
547 Invisalign® SmartTrack® Material. *Materials (Basel)* 13, 5311.  
548 <https://doi.org/10.3390/ma13235311>

549 Orilisi, G., Monterubbianesi, R., Notarstefano, V., Tosco, V., Vitiello, F., Giuliani, G., Putignano,  
550 A., Orsini, G., 2021. New insights from Raman MicroSpectroscopy and Scanning Electron  
551 Microscopy on the microstructure and chemical composition of vestibular and lingual surfaces in  
552 permanent and deciduous human teeth. *Spectrochim Acta A Mol Biomol Spectrosc* 260, 119966.  
553 <https://doi.org/10.1016/j.saa.2021.119966>

554 Orsini, G., Orilisi, G., Notarstefano, V., Monterubbianesi, R., Vitiello, F., Tosco, V., Belloni, A.,  
555 Putignano, A., Giorgini, E., 2021. Vibrational Imaging Techniques for the Characterization of Hard  
556 Dental Tissues: From Bench-Top to Chair-Side. *Applied Sciences* 11, 11953.  
557 <https://doi.org/10.3390/app112411953>

558 Pacheco-Pereira, C., Brandelli, J., Flores-Mir, C., 2018. Patient satisfaction and quality of life  
559 changes after Invisalign treatment. *Am J Orthod Dentofacial Orthop* 153, 834–841.  
560 <https://doi.org/10.1016/j.ajodo.2017.10.023>

561 Papadopoulou, A.K., Cantele, A., Polychronis, G., Zinelis, S., Eliades, T., 2019. Changes in  
562 Roughness and Mechanical Properties of Invisalign® Appliances after One- and Two-Weeks Use.  
563 *Materials (Basel)* 12. <https://doi.org/10.3390/ma12152406>

564 Park, H., Park, B., 2021. Review of Microplastic Distribution, Toxicity, Analysis Methods, and  
565 Removal Technologies. *Water* 13, 2736. <https://doi.org/10.3390/w13192736>

566 Paul, M.B., Stock, V., Cara-Carmona, J., Lisicki, E., Shopova, S., Fessard, V., Braeuning, A., Sieg,  
567 H., Böhmert, L., 2020. Micro- and nanoplastics – current state of knowledge with the focus on oral  
568 uptake and toxicity. *Nanoscale Adv.* 2, 4350–4367. <https://doi.org/10.1039/D0NA00539H>

569 Prata, J.C., 2018. Airborne microplastics: Consequences to human health? *Environ Pollut* 234, 115–  
570 126. <https://doi.org/10.1016/j.envpol.2017.11.043>

571 Prata, J.C., da Costa, J.P., Lopes, I., Duarte, A.C., Rocha-Santos, T., 2020. Environmental exposure  
572 to microplastics: An overview on possible human health effects. *Science of The Total Environment*  
573 702, 134455. <https://doi.org/10.1016/j.scitotenv.2019.134455>

574 Putrino, A., Barbato, E., Galluccio, G., 2021. Clear Aligners: Between Evolution and Efficiency-A  
575 Scoping Review. *Int J Environ Res Public Health* 18, 2870. <https://doi.org/10.3390/ijerph18062870>

576 Ragusa, A., Notarstefano, V., Svelato, A., Belloni, A., Gioacchini, G., Blondeel, C., Zucchelli, E.,  
577 De Luca, C., D'Avino, S., Gulotta, A., Carnevali, O., Giorgini, E., 2022. Raman Microspectroscopy  
578 Detection and Characterisation of Microplastics in Human Breastmilk. *Polymers* 14, 2700.  
579 <https://doi.org/10.3390/polym14132700>

580 Ragusa, A., Svelato, A., Santacroce, C., Catalano, P., Notarstefano, V., Carnevali, O., Papa, F.,  
581 Rongioletti, M.C.A., Baiocco, F., Draghi, S., D'Amore, E., Rinaldo, D., Matta, M., Giorgini, E.,  
582 2021. Plasticenta: First evidence of microplastics in human placenta. *Environment International*  
583 146, 106274. <https://doi.org/10.1016/j.envint.2020.106274>

584 Ribeiro Claro, P., Nolasco, M., Araújo, C., 2016. Characterization of Microplastics by Raman  
585 Spectroscopy, in: *Comprehensive Analytical Chemistry*.  
586 <https://doi.org/10.1016/bs.coac.2016.10.001>

587 Robertson, L., Kaur, H., Fagundes, N.C.F., Romanyk, D., Major, P., Flores Mir, C., 2020.  
588 Effectiveness of clear aligner therapy for orthodontic treatment: A systematic review. *Orthod*  
589 *Craniofac Res* 23, 133–142. <https://doi.org/10.1111/ocr.12353>

590 Rocha-Santos, T., Duarte, A.C., 2015. A critical overview of the analytical approaches to the  
591 occurrence, the fate and the behavior of microplastics in the environment. *TrAC Trends in*  
592 *Analytical Chemistry* 65, 47–53. <https://doi.org/10.1016/j.trac.2014.10.011>

593 Romano, A., Di Spirito, F., Amato, A., Ferraro, G.A., Dipalma, G., Xhajanka, E., Serpico, R.,  
594 Inchingolo, F., Contaldo, M., 2022. Dental Microstructural Imaging: From Conventional Radiology  
595 to In Vivo Confocal Microscopy. *Applied Sciences* 12, 10654.  
596 <https://doi.org/10.3390/app122010654>

597 Ryokawa, H., Miyazaki, Y., Fujishima, A., Miyazaki, T., Maki, K., 2006. The mechanical  
598 properties of dental thermoplastic materials in a simulated intraoral environment. *Orthodontic*  
599 *Waves* 65, 64–72. <https://doi.org/10.1016/j.odw.2006.03.003>

600 Schwabl, P., Köppel, S., Königshofer, P., Bucsics, T., Trauner, M., Reiberger, T., Liebmann, B.,  
601 2019. Detection of Various Microplastics in Human Stool: A Prospective Case Series. *Ann Intern*  
602 *Med* 171, 453–457. <https://doi.org/10.7326/M19-0618>

603 Shi, B., Patel, M., Yu, D., Yan, J., Li, Z., Petriw, D., Pruyn, T., Smyth, K., Passeur, E., Miller,  
604 R.J.D., Howe, J.Y., 2022. Automatic quantification and classification of microplastics in scanning  
605 electron micrographs via deep learning. *Science of The Total Environment* 825, 153903.  
606 <https://doi.org/10.1016/j.scitotenv.2022.153903>

607 Song, Y.K., Hong, S.H., Jang, M., Han, G.M., Rani, M., Lee, J., Shim, W.J., 2015. A comparison of  
608 microscopic and spectroscopic identification methods for analysis of microplastics in environmental  
609 samples. *Marine Pollution Bulletin* 93, 202–209. <https://doi.org/10.1016/j.marpolbul.2015.01.015>

610 Tamargo, A., Molinero, N., Reinoso, J.J., Alcolea-Rodriguez, V., Portela, R., Bañares, M.A.,  
611 Fernández, J.F., Moreno-Arribas, M.V., 2022. PET microplastics affect human gut microbiota  
612 communities during simulated gastrointestinal digestion, first evidence of plausible polymer  
613 biodegradation during human digestion. *Sci Rep* 12, 528. <https://doi.org/10.1038/s41598-021-04489-w>

614

615 Tamburrino, F., D'Antò, V., Bucci, R., Alessandri-Bonetti, G., Barone, S., Razionale, A.V., 2020.  
616 Mechanical Properties of Thermoplastic Polymers for Aligner Manufacturing: In Vitro Study. *Dent*  
617 *J (Basel)* 8, 47. <https://doi.org/10.3390/dj8020047>

618 Tartaglia, G.M., Mapelli, A., Maspero, C., Santaniello, T., Serafin, M., Farronato, M., Caprioglio,  
619 A., 2021. Direct 3D Printing of Clear Orthodontic Aligners: Current State and Future Possibilities.  
620 *Materials (Basel)* 14, 1799. <https://doi.org/10.3390/ma14071799>

621 Torsello, F., D'Amico, G., Staderini, E., Marigo, L., Cordaro, M., Castagnola, R., 2022. Factors  
622 Influencing Appliance Wearing Time during Orthodontic Treatments: A Literature Review.  
623 *Applied Sciences* 12, 7807. <https://doi.org/10.3390/app12157807>

624 Tosco, V., Vitiello, F., Furlani, M., Gatto, M.L., Monterubbianesi, R., Giuliani, A., Orsini, G.,

625 Putignano, A., 2021. Microleakage Analysis of Different Bulk-Filling Techniques for Class II  
626 Restorations:  $\mu$ -CT, SEM and EDS Evaluations. *Materials* 14, 31.  
627 <https://doi.org/10.3390/ma14010031>

628 Triebkorn, R., Braunbeck, T., Grummt, T., Hanslik, L., Huppertsberg, S., Jekel, M., Knepper, T.P.,  
629 Kraiss, S., Müller, Y.K., Pittroff, M., Ruhl, A.S., Schmieg, H., Schür, C., Strobel, C., Wagner, M.,  
630 Zumbülte, N., Köhler, H.-R., 2019. Relevance of nano- and microplastics for freshwater  
631 ecosystems: A critical review. *TrAC Trends in Analytical Chemistry* 110, 375–392.  
632 <https://doi.org/10.1016/j.trac.2018.11.023>

633 Urban, R.M., Jacobs, J.J., Tomlinson, M.J., Gavrilovic, J., Black, J., Peoc'h, M., 2000.  
634 Dissemination of wear particles to the liver, spleen, and abdominal lymph nodes of patients with hip  
635 or knee replacement. *J Bone Joint Surg Am* 82, 457–476. <https://doi.org/10.2106/00004623-200004000-00002>

637 Vitiello, F., Tosco, V., Monterubbianesi, R., Orilisi, G., Gatto, M.L., Sparabombe, S., Memé, L.,  
638 Mengucci, P., Putignano, A., Orsini, G., 2022. Remineralization Efficacy of Four Remineralizing  
639 Agents on Artificial Enamel Lesions: SEM-EDS Investigation. *Materials (Basel)* 15, 4398.  
640 <https://doi.org/10.3390/ma15134398>

641 Wang, W., Wang, J., 2018. Investigation of microplastics in aquatic environments: An overview of  
642 the methods used, from field sampling to laboratory analysis. *Trends in analytical chemistry*.

643 Wang, Z.-M., Wagner, J., Ghosal, S., Bedi, G., Wall, S., 2017. SEM/EDS and optical microscopy  
644 analyses of microplastics in ocean trawl and fish guts. *Science of The Total Environment* 603–604,  
645 616–626. <https://doi.org/10.1016/j.scitotenv.2017.06.047>

646 Weir, T., 2017. Clear aligners in orthodontic treatment. *Aust Dent J* 62 Suppl 1, 58–62.  
647 <https://doi.org/10.1111/adj.12480>

648 Wieland, S., Balmes, A., Bender, J., Kitzinger, J., Meyer, F., Ramsperger, A.F., Roeder, F.,  
649 Tengelmann, C., Wimmer, B.H., Laforsch, C., Kress, H., 2022. From properties to toxicity:  
650 Comparing microplastics to other airborne microparticles. *Journal of Hazardous Materials* 428,  
651 128151. <https://doi.org/10.1016/j.jhazmat.2021.128151>

652 Willert, H.G., Semlitsch, M., 1996. Tissue reactions to plastic and metallic wear products of joint  
653 endoprostheses. *Clin Orthop Relat Res* 4–14.

654 Xu, H., Dinsdale, D., Nemery, B., Hoet, P.H.M., 2003. Role of residual additives in the cytotoxicity  
655 and cytokine release caused by polyvinyl chloride particles in pulmonary cell cultures. *Toxicol Sci*  
656 72, 92–102. <https://doi.org/10.1093/toxsci/kfg003>

657 Yang, X., Man, Y.B., Wong, M.H., Owen, R.B., Chow, K.L., 2022. Environmental health impacts  
658 of microplastics exposure on structural organization levels in the human body. *Science of The Total*  
659 *Environment* 825, 154025. <https://doi.org/10.1016/j.scitotenv.2022.154025>

660 Yee, M.S.-L., Hii, L.-W., Looi, C.K., Lim, W.-M., Wong, S.-F., Kok, Y.-Y., Tan, B.-K., Wong, C.-  
661 Y., Leong, C.-O., 2021. Impact of Microplastics and Nanoplastics on Human Health. *Nanomaterials*  
662 (Basel) 11, 496. <https://doi.org/10.3390/nano11020496>

663 Zhu, M., Nie, G., Meng, H., Xia, T., Nel, A., Zhao, Y., 2013. Physicochemical Properties  
664 Determine Nanomaterial Cellular Uptake, Transport, and Fate. *Acc. Chem. Res.* 46, 622–631.  
665 <https://doi.org/10.1021/ar300031y>  
666