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

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ABSTRACT

Clear orthodontic aligners have recently seen increasing popularity. The thermoplastic materials present several advantages, even if it is known that all plastic products can be subjected to environmental and mechanical degradation, leading to the release of microplastics (MPs). Their ingestion could cause oxidative stress and inflammatory lesions. This study aims to evaluate the potential detachment of MPs by clear aligners due to mechanical friction simulated with a 7-days protocol in artificial saliva. The study was performed on orthodontic clear aligners from different manufacturers: Alleo (**AL**); Flexi Ligner (**FL**); F22 Aligner (**F22**); Invisalign (**INV**); Lineo (**LIN**); ArcAngel (**ARC**), and Ortobel Aligner (2). For each group, two aligners were immersed in artificial saliva for 7 days and stirred for 5 hours/day, simulating the physiological teeth mechanical friction. After 7 days, the artificial saliva was filtered; filters were analyzed by Raman Microspectroscopy (RMS) and Scanning Electron Microscopy (SEM), respectively to chemically identify the polymeric matrix and to measure the number and size of the detected MPs. MPs were evaluated in terms of chemical composition, number, and size. RMS spectra revealed that **AL**, **FL**, **LIN**, **ARC**, and **OR** aligners were composed by polyethylene terephthalate, while **F22** and **INV** ones by polyurethane. SEM analysis showed that the highest number of MPs was found in **ARC** and the lowest in **INV** ($p<0.05$). As regards MPs' size, no statistically significant difference was found among groups, with most MPs ranging from 5 to 20 µm. Noteworthy, a highly significant correlation ($p<0.0001$) was highlighted between the distribution of MPs size and the different typologies of aligners. This in vitro study highlighted for the first the detachment of MPs from clear aligners due to mechanical friction. This evidence may represent a great concern in the clinical practice since it could impact human general health.

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

1. Introduction

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

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

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

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

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

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

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

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

2. Materials and methods

2.1 Materials

The orthodontic clear aligners, derived from the same STL file, were provided by the following manufacturers: Alleo (**AL**, Digital Service Leone s.r.l, Florence, Italy); Flexi Ligner (**FL**, Roma, Italy); F22 Aligner (**F22**, Sweden & Martina Spa, Padova, Italy); Invisalign (**INV**, Align Technology, Mexico); Lineo (**LIN**, Micerium Lab, Milan, Italy); ArcAngel (**ARC**, Network Gruppo Dextra, Modena, Italy), and Ortobel Aligner (**OR**, Bergamo, Italy).

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

2.2 Samples' treatment

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

2.3 Raman Microspectroscopy analysis

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

2.4 Scanning Electron Microscopy analysis

SEM analysis was performed at the Centre for Electron Microscopy – CISMIN (Department of Materials, Environmental Science and Urban Planning, Polytechnic University of Marche, Ancona, Italy). From the same filters analyzed by RMS, supposing a homogeneous distribution of fragments, a representative circular portion with a diameter of *ca.* 20 mm was cropped (Hidalgo-Ruz et al., 2012); more in detail, the original filter had an area of *ca.* 1734.1 mm^2, while the cropped filter of *ca.* 314.0 mm^2. The cropped filters were mounted on aluminum stubs, sputter-coated with gold and observed by a TESCAN VEGA 3 LMU scanning electron microscope. SEM operated at 10 kV and at variable working distance with secondary electron detector (SE).

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

2.5 Quality Assurance and Control

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

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

2.6 Statistical Analysis

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

3. Results

Filters from all the experimental groups were first analyzed by RMS; then, they were cut, and the cropped circular portions were submitted to SEM evaluation. The details, including the chemical composition, number, and size, of all the microparticles detected in the three replicates, are reported in Table 1. As regards the number of MPs, it represents the number of microparticles found in the cut filter portions (diameter *ca.* 20 mm). It is noteworthy that both in environmental and procedural 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 (µm)	Smallest (µm)	Largest (µm)
AL	PET	#1	12	13.72 ± 7.07	3.76	22.11
		#2	14	16.16 ± 8.67	3.41	28.31
		#3	12	16.09 ± 7.25	4.78	28.9
FL	PET	#1	10	20.37 ± 7.23	7.77	36.39
		#2	8	20.12 ± 10.74	9.18	28.36
		#3	12	19.29 ± 11.76	8.34	45.70
F22	PU	#1	11	16.12 ± 9.41	4.55	31.58
		#2	10	19.49 ± 12.69	4.83	38.28
		#3	12	18.80 ± 9.92	8.97	34.91
INV	PU	#1	7	16.64 ± 9.66	3.13	31,97
		#2	5	12.12 ± 5.70	3.96	18.57
		#3	7	15.81 ± 11.37	3.85	34.20

LIN	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
ARC	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
OR	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.

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

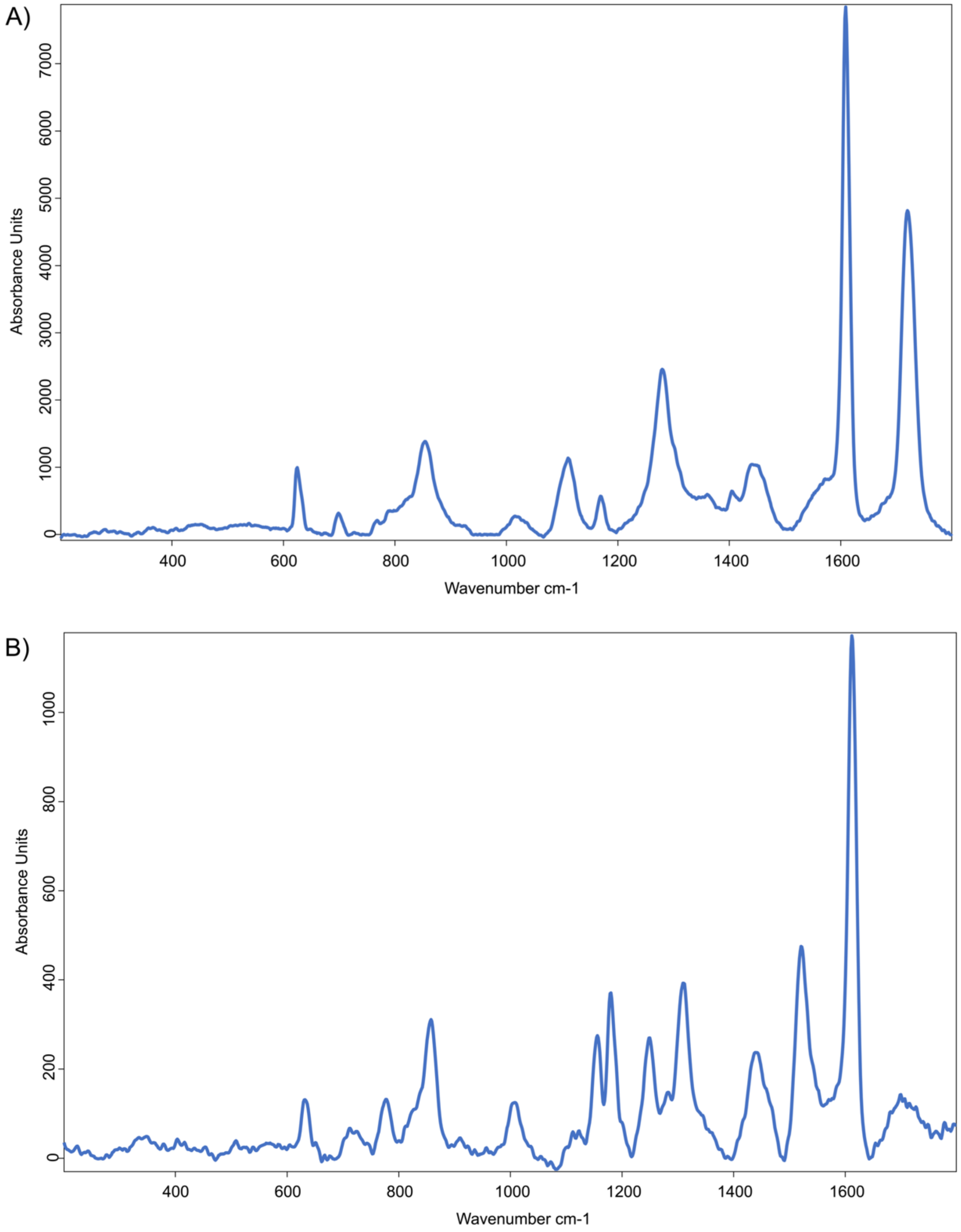


Fig 1. Representative RMS spectra collected on the microparticles detached from the aligners. Spectra (A) and (B) were ascribable respectively to polyethylene terephthalate and polyurethane. Spectrum (A): Alleo (**AL**), Flexi Ligner (**FL**), Lineo (**LIN**), ArcAngel (**ARC**), and Ortobel (**OR**).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

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

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

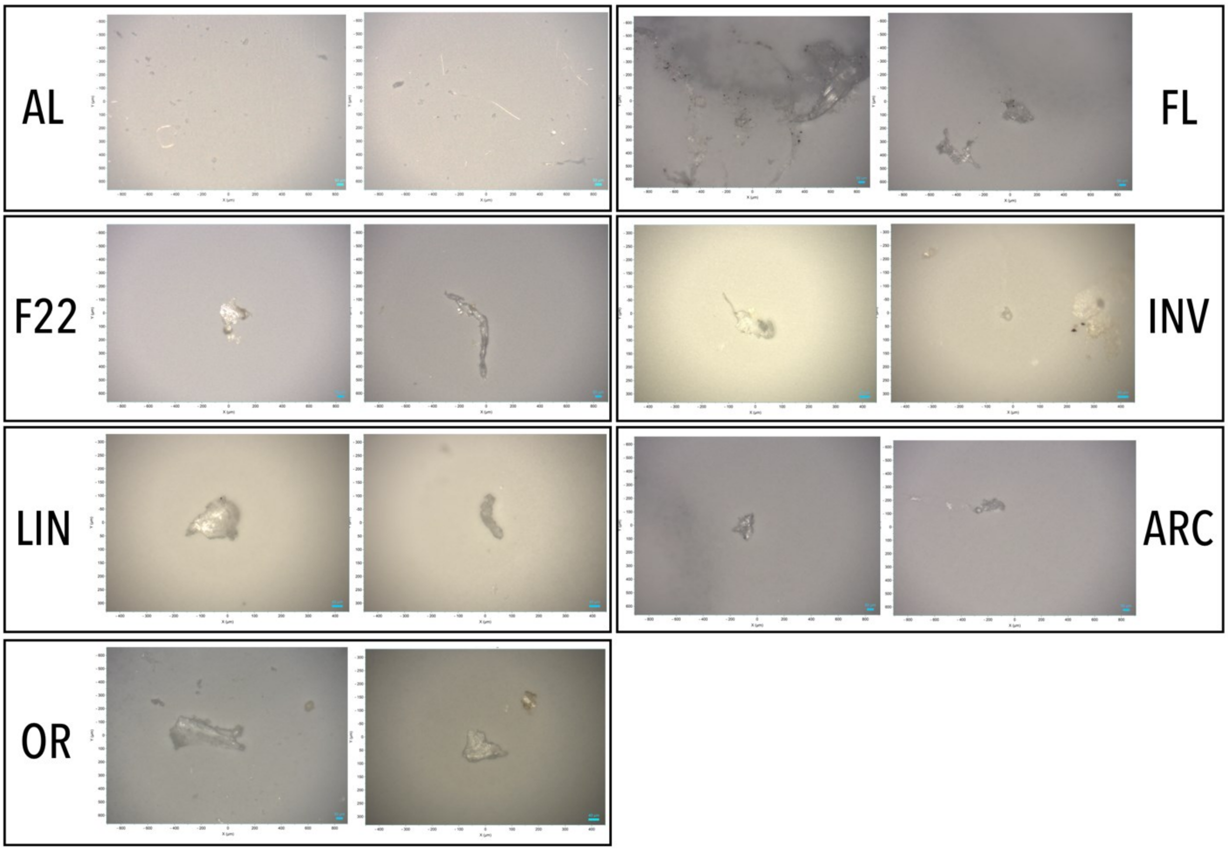


Fig. 2. Microphotographs of some selected microparticles detached from the following aligners: **AL**: Alleo; **FL**: Flexi Ligner; **F22**: F22 Aligner; **INV**: Invisalign; **LIN**: Lineo; **ARC**: ArcAngel; **OR**: Ortobel Aligner (100x magnification, Olympus MPLAN100×/0.90)

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

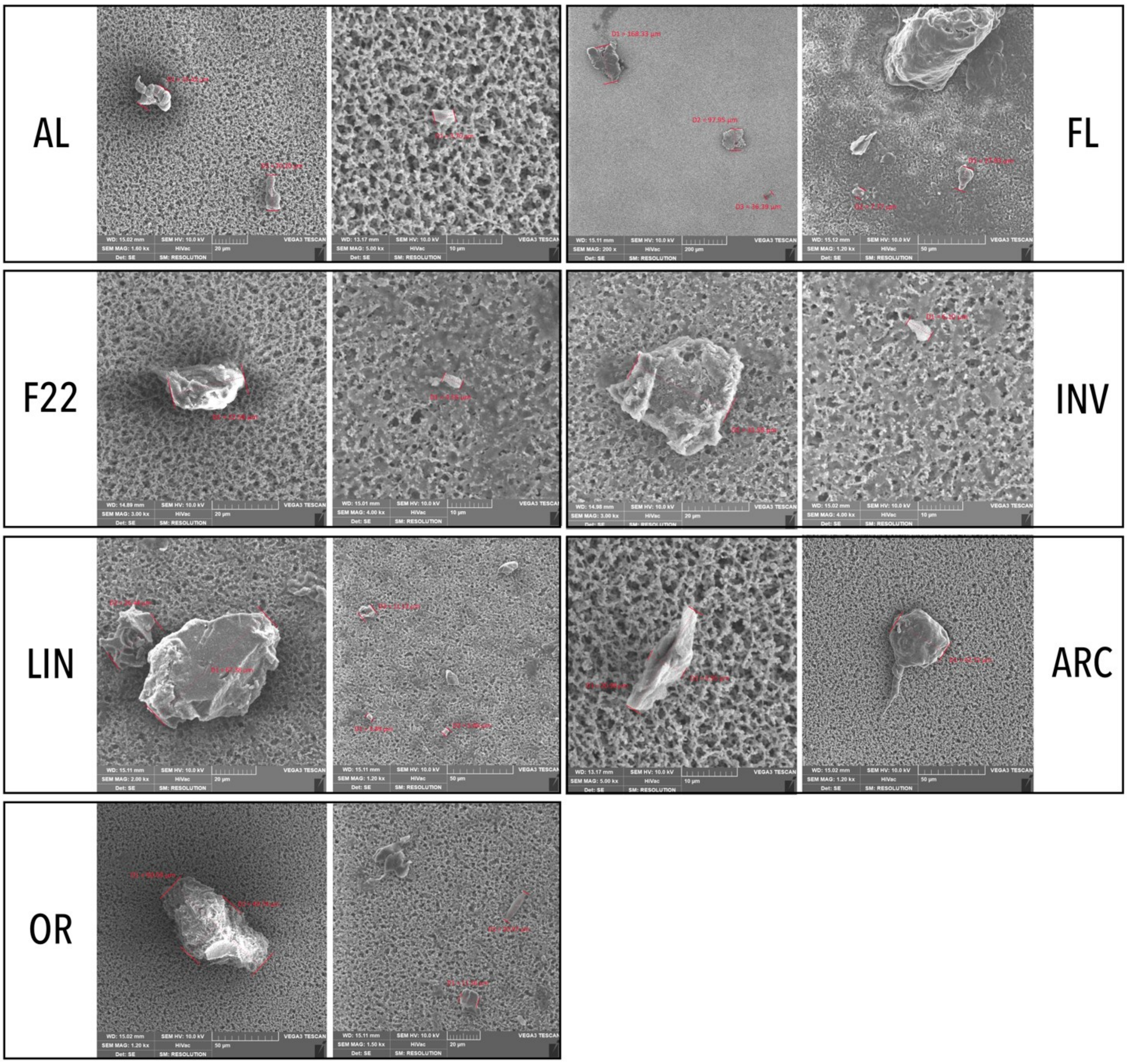
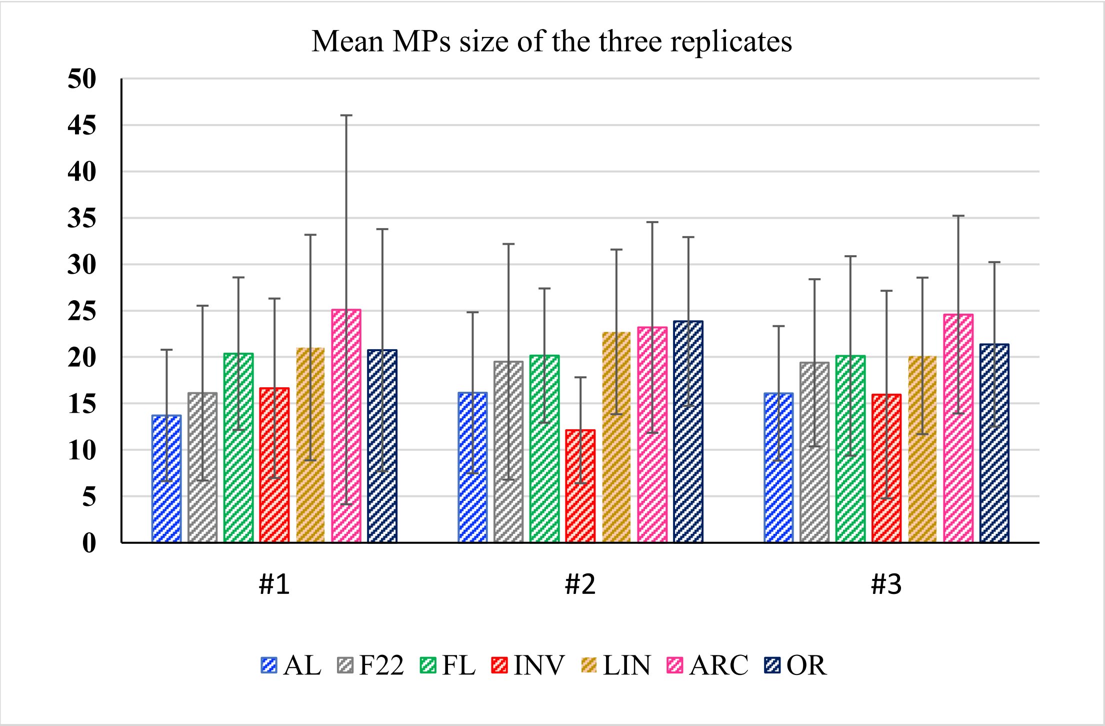


Fig. 3. Scanning electron micrographs collected at different magnifications on some selected MPs detached from the following aligners: **AL**: Alleo; **FL**: Flexi Ligner; **F22**: F22 Aligner; **INV**: Invisalign; **LIN**: Lineo; **ARC**: ArcAngel; **OR**: Ortobel. For each micrograph, the sizes of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

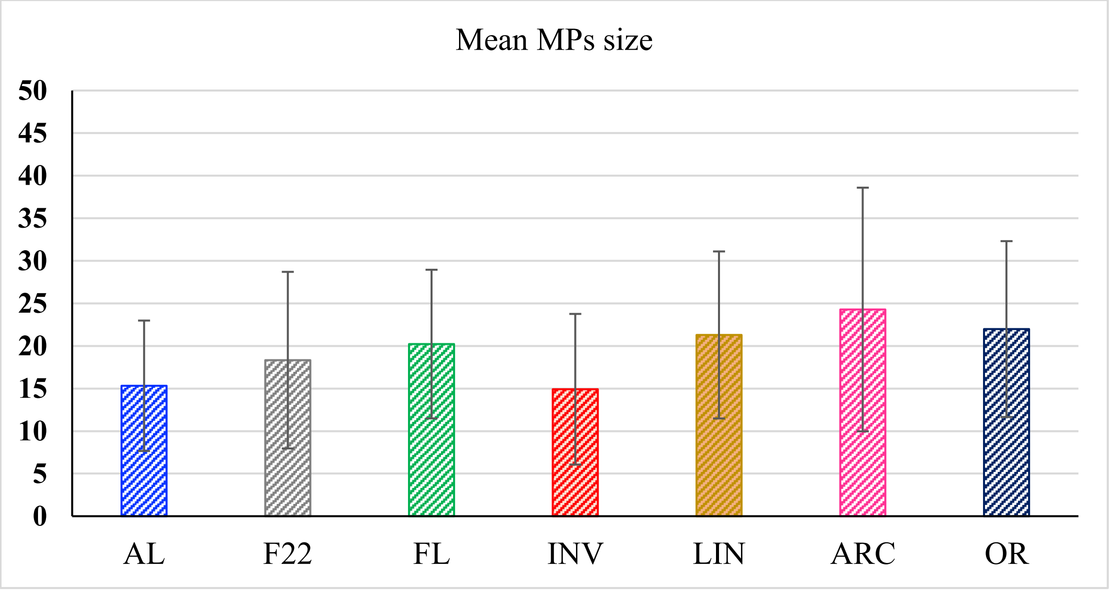
The average number of MPs, found in the different aligners and derived from the three replicates, revealed statistically significant differences between the tested groups ($p<0.05$). In particular, more than 10 MPs were counted in **AL** (13 ± 1), **F22** (11 ± 1), **FL** (10 ± 2), **LIN** (15± 2), **ARC**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

11 in **OR**; N. 99 ±11 in **ARC**; N. 33 ± 6 in **INV**. Hence, the highest number was found in **ARC** and the lowest one in **INV**.

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



(A)



(B)

Fig. 4. (A) Mean size (µm) and standard deviation of MPs detected in the experimental groups, subdivided in the three replicates (#1, #2, #3); (B) mean size (µm) 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

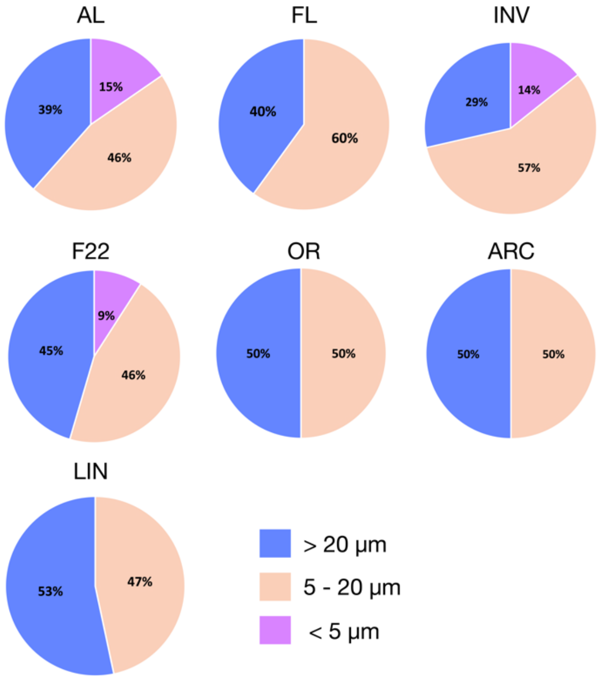


Fig. 5. Distribution in percentage (%) of the mean MPs sizes of the three replicates, subdivided into 3 ranges (< 5 µm, 5-20 µm and > 20 µm), for each group: **AL** (Alleo); **FL** (Flexi Ligner); **F22** (F22 Aligner); **INV** (Invisalign); **LIN** (Lineo); **ARC** (ArcAngel); **OR** (Ortobel Aligner).

4. Discussion

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

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

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

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

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

Currently, there is growing scientific evidence about MPs in humans, with an estimated total intake of 39-52 thousand MPs per person per year, mainly through ingestion (Cox et al., 2019; Prata, 2018; Prata et al., 2020). According to the scientific literature, the primary health effects of ingested MPs are triggered from the digestive system, causing direct damage not only at local level, such as irritation or intestinal dysbiosis, but also at systemic level (Tamargo et al., 2022; Yee et al., 2021). To date, the changes of MPs during gastrointestinal digestion or colonic fermentation are scarcely explored. However, a recent study provided scientific evidence of modifications and potential effects of MPs during their passage through the digestive tract (Tamargo et al., 2022). Indeed, authors reported that PET MPs during gastrointestinal digestion showed structural changes, suggesting a potential biodegradation probably driven by colonic microbiota, supporting the existence of an interaction between the colonic microbiota and PET MPs particles. Although there are few experimental researches on MPs metabolism in the human body, studies agree that their uptake is influenced by their shape and size (Triebskorn et al., 2019; Wieland et al., 2022). In particular, size represents a crucial factor, with larger particles requiring active endocytosis, while very small particles being able to passively cross membranes (Kettiger et al., 2013; Triebskorn et al., 2019; Yang et al., 2022). Indeed, particles > 20 µm are likely excreted from the gastrointestinal tract (Schwabl et al., 2019; Wieland et al., 2022). Conversely, microparticles ranging from 5 µm to 20 µm, at the gastrointestinal level, may pass through the epithelium by endocytosis mechanisms or by paracellular diffusion. After that, MPs are translocated by dendritic cells through the lymphatic circulation and reach the circulatory system (Prata et al., 2020). As regards our results, microparticles with a diameter of 5 - 20 µm were found in all the attested aligners and represented the largest group, with a percentage higher than 50%, except that for **F22** and **LIN** (36% and 46%, respectively). A percentage range of 30-50% was detected for MPs > 20 µm in all the aligners, while MPs < 5 µm were detected only in **AL** (17%), **F22** (18%) and **INV** (14%). In this context, it needs to be considered if these MPs could pass the gastrointestinal tract through para and/or transcellular manner. Florence et al. reported that the uptake of MPs by M-cells into the Peyer's patches (lymphoid follicles in the small intestine) plays a significant role (Florence, 1997). Indeed, the presence of MPs could cause aggregations of macrophages, granulation tissue and foreign body response, with inflammation and oxidative stress (Paul et al., 2020; Urban et al., 2000; Willert and Semlitsch, 1996). In this light, it is suggested to use this type of orthodontic treatment with caution in growing children.

Recent studies showed that the leaching of monomers from MPs could contribute to their toxicity (Mastrangelo et al., 2002; Xu et al., 2003). MPs deriving from **F22** and **INV** aligners appeared as aggregates of microspheres, and, hence, they could lead to a further detachment of microparticles with smaller diameter and with higher toxicity into the gastrointestinal tract. Moreover, in **FL**, **INV** and **ARC** aligners, some of the identified MPs appeared blue or black pigmented. This finding, probably related to the ink used to identify the aligner, could be explained by the fact that these areas may be less resistant to mechanical friction, leading to easier detachment of the MPs.

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

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

5. Conclusions

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

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