Effect of salivary fluid characteristics on the physical features of in vitro bread bolus: from the absence of saliva to artificially simulated hypersalivation

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Effect of salivary fluid characteristics on the physical features of *in vitro* bread bolus: from the absence of saliva to artificially simulated hypersalivation

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Keywords

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Highlights

- Saliva incorporation affects particle size and fluid content of in vitro bread bolus
- An excess of saliva fluid leads to bread boluses constituted by smaller particles
- A lack of saliva to form the bread bolus leads to extremely large particles
- The enzymatic activity of α-amylase favours bread particle size reduction
- Saliva temperature does not significantly affect bread particle size reduction

Abstract

Saliva facilitates food oral processing, bolus formation, swallowing, and sensory perception, in addition to contributing to oral health and phonation. Ageing, health affections, and polymedication are among many causes altering salivary production, modifying the mastication process, the food impregnation ratio, and in turn altering the characteristics of the bolus, swallowing, and digestion. In this *in vitro* work, using the AM^2 masticator apparatus, which replicates the mechanical actions taking place while chewing solid foods and produces realistic food bolus in various oral conditions, we investigated the effect of salivary fluid characteristics, i.e., composition, quantity (from absence to hypersalivation), temperature, and enzymatic action, on the physical characteristics (i.e., particle size distribution (*PSD*), bolus mass, salivary fluid content) of *in vitro* boluses of Traditional French baguette.

A ready-to-swallow bolus of baguette displayed on average a d_{50} value (median particle size by mass) of 4.1 ± 0.4 mm, with saliva fluid constituting ~35% of the final bolus mass. The absence of saliva in mouth led to a deficient oral processing, forming bread boluses constituted by extremely big particles (ca. 80% of particles had a size >7.1 mm) that likely cannot be swallowed safely. On the contrary, an excess of saliva favoured an excessive breaking down of bread, leading to bread boluses constituted by smaller particles than those formed under healthy salivary conditions (d_{50} decreased from 4.1 mm to 3.1 mm), having a

higher salivary fluid content (+10%). On the other hand, the salivary fluid temperature did not affect *PSD*, d_{50}, bolus mass, or salivary fluid content of *in vitro* bread boluses, however, the addition of human salivary α-amylase did, favouring particle size reduction (d_{50} decreased to 2.6 mm). Therefore, beyond the correlation between bolus hydration by saliva and food properties such as hardness and moisture content, our findings indicate that the quantity of salivary fluid present in the oral cavity and the enzymatic activity of salivary α-amylase during bread mastication significantly influence both the particle size distribution and the fluid content of bread boluses, ultimately determining the physical properties of the bolus and, therefore, potentially impacting the subsequent swallowing process.

1. Introduction

Food oral processing (FOP) commences with the initial bite, progresses through mastication with a sequence of food structural and physicochemical transformations, and ends with the act of swallowing. FOP involves all oral components and encompasses all activities contributing to food management and bolus formation, including sensory perception, muscular contractions, movements of the jaw and tongue, and the provision of saliva (Chen, 2009; Koç et al., 2013; van der Bilt et al., 2006).

During mastication, the structure of solid foods undergoes notable changes, with the most apparent being a reduction in size, resulting in numerous fragments that are swiftly moistened by saliva. The provision of saliva, tongue movements, and the act of mastication are interconnected functions that facilitate food fragmentation and particle aggregation, allowing the creation of a lubricated and softened food bolus that ensures safe and comfortable swallowing (Mosca & Chen, 2017; Muñoz-Núñez et al., 2023). More than 99% of saliva is water, along with electrolytes, enzymes, other proteins including large molecular (e.g., mucins) and small molecular weight proteins (e.g., lactoferrin and amylase), and nitrogenous compounds such as urea and ammonia (Pedersen et al., 2018). Saliva serves as a lubricant within the oral cavity, covering its interior surfaces, eliminating debris, dissolving taste compounds, capturing fragrances, and enzymatically breaking down various substances (Dantas et al., 1990; Humphrey & Williamson, 2001; Zussman et al., 2007). During chewing, oral surfaces must be lubricated for preventing tissue irritation and frictional damage, and for facilitating food oral processing and particles and bolus transportation (Christersson et al., 2000; Glantz & Friberg, 1970; Schwarz, 1987).

Over the past few decades, several hypotheses have been postulated to explain the mechanisms that trigger the swallowing of food boluses, considering diverse factors such as the degree of lubrication and structure of the bolus, its maximum cohesive force, particle size,

and other sensory cues that may influence the process such as stickiness (Hutchings & Lillford, 1988; Jalabert-Malbos et al., 2007a; Peyron et al., 2004, 2011; Prinz & Lucas, 1997). Extensive research has focused on the function of saliva during the oral phase, primarily for its role in enhancing the rheological properties of the bolus (e.g., cohesiveness, plasticity, stickiness; Humphrey & Williamson, 2001; Peyron et al., 2011; Stokes & Davies, 2007). For example, the elasticity and extensional properties of a model bolus were shown to strongly influence its flow during *in vitro* swallowing (Marconati & Ramaioli, 2020). Consequently, the distinct extensional properties of human saliva are also anticipated to exert a significant impact during the process of swallowing. In addition to its rheological impact, saliva plays a vital biochemical role in initiating the digestion of starch, a phenomenon frequently observed during the chewing process of various types of bread (Gao et al., 2015; Gao & Zhou, 2021; Hoebler et al., 1998; Jourdren et al., 2016; Le Bleis et al., 2016; Pentikäinen et al., 2014; Tournier et al., 2012). Its primary function involves breaking down intricate carbohydrates into simpler sugars, aiding their absorption. Among the enzymes found in saliva, α-amylase stands out as the most abundant. Its enzymatic properties, such as its secretion by salivary glands, concentration levels, amylolytic activity, gender and age-related distinctions, vulnerability to inactivation in acidic gastric conditions, optimal temperature for activity (inactivation below 32°C and denaturation above 37°C), influence on taste perception, and behaviour in particular environments, have all become more precisely characterised (Al-Manei et al., 2020; Jain et al., 2020; Zhang et al., 2022; to name a few).

Large variations exist in the oral state quality among subjects, where an impaired salivary function can obviously alter FOP and more generally oral protection and maintenance. A salivary gland dysfunction can be described either as a reduction or an increase in salivary production. These dysfunctions are generally described as a consequence of ageing, various diseases, medications or medical therapies (Liu et al., 2012; Pedersen et al., 2018). The

subjective sensation of dry mouth, namely xerostomia, is often associated with a decrease in saliva flow and altered saliva composition, a condition in which the salivary glands do not produce enough saliva or in bad quality to keep the mouth moist (Escobar & Aitken-Saavedra, 2018; Xu et al., 2019). Approximately 30% of patients over 60 experience oral dryness, increasing to 60% in long-term care facilities (Escobar & Aitken-Saavedra, 2018; Sreebny, 2000). On the contrary an excessive amount of saliva could also be frequently observed in case of neurological diseases, oral inflammation, medication, gastroesophageal reflux or toxin exposure for example. Drooling may be a consequence of hypersalivation (sialorrhea) but more frequently due to impairment in neuromuscular control or swallowing disorders (dysphagia). Among adults, Parkinson's disease (prevalence 205.89 per 100.000 inhabitants) is the most common cause of sialorrhea (70–80%; Lakraj et al., 2013), showing a prevalence increase of 62.13% among those over 40 (Orozco et al., 2020). Approximately 8% of the world's population suffers from dysphagia (Cichero et al., 2013), having a prevalence over age 50 between 15% and 22%, being even higher in nursing homes and assisted living facilities (40% - 60%; Aslam & Vaezi, 2013). People suffering from hyposalivation, sialorrhea or dysphagia due to saliva impairment present higher risk of saliva or food fragments aspiration, choking, aspiration pneumonia, reduced food intake, malnutrition, dehydration, morbidity, and mortality (Beck et al., 2018). This is particularly concerning in an ageing society where 1 in 6 people will be over 65 by 2050 (United Nations, 2020).

For those populations mentioned above, the potential impact of changes in saliva production during chewing on food oral processing and the resulting characteristics of the formed bolus may have significant implications. So far, no prior studies have examined the influence of saliva features on the granulometric properties of the bolus, particularly in the state authorising its swallowing. In this work, we investigate the effect that the salivary fluid type, quantity in mouth (from the absence of saliva to artificially simulated hypersalivation),

its salivary amylase action may have on the granulometric characteristics of *in vitro* Traditional French baguette boluses, using the Artificial Masticatory Advanced Machine (AM^2; Peyron et al., 2019).

2. Materials & Methods

2.1 Model bread: Traditional French baguette

Traditional French baguette is a long and thin loaf of bread. Its artisanal manufacture is regulated by a French decree authorizing the exclusive use of white wheat flour, water, yeast, and salt, without any additive. During its manufacture, it is subjected to a long fermentation. A traditional French baguette is approx. composed of 57% carbohydrates, 30% water, 9% proteins, 1% lipids and 3% fibres. Before testing, freshly baked baguettes were purchased from a local bakery on a daily basis. From a 2 cm thick slice of baguette, 1/4 is sized and weighted until it has a mass of approximately 2.6 ± 0.1 g.

2.2 Artificial salivary fluids

Several oral lubricants, varying in terms of composition and volume (quantity), were tested in this work to simulate the effect of normal salivation with or without human salivary α-amylase, hypersalivation, and an absence of saliva on the granulometric characteristics of the food bolus. The experimental conditions applied are detailed in **Table 1**. The effect of ambient salivary fluid temperature (20°C), which is frequently set for *in vitro* food oral processing experiments, on bolus granulometry was compared to *in vivo* mouth temperature (37°C), which is the temperature required *in vitro* when enzyme is used. The artificial saliva (AS) was composed of a "basic salivary fluid" (BSF) containing α-amylase from human saliva. The BSF shows representative chemical (e.g., some mineral and glycoproteic contents) and rheological (e.g., shear viscosity) features of those of human saliva (Roger-Leroi et al., 2012). Its composition consisted of a mixture of 5.208 g $NaHCO_3$, 1.369 g $K_2HPO_4.3H_2O$,

0.877 g NaCl, 0.477 g KCl, 0.441g $CaCl_2.2H_2O$, and 2.16 g mucin (from porcine stomach, Type II; Sigma®) in 1L of milli-Q water, adjusted to pH 6.95 with HCl 1N. To prepare AS, 92 U/mL of α-amylase (from human saliva, A1031-5KU, Sigma®) were incorporated; the activity per unit of saliva was chosen based on the data reported in (Mandel et al., 2010); there is no change in apparent viscosity when amylase is incorporated (Roger-Leroi et al., 2012). As α-amylase activity is dependent on temperature, AS was kept at 37°C (body temperature) before its introduction into the mastication chamber.

When AS was used, α-amylase deactivation was also carried out just after bolus collection to verify that its enzymatic action does not lead to any post-mastication alterations of bolus characteristics; the bolus was soaked in 10 mL of the 0.01M HCl (pH 2), being gently stirred to favour enzyme deactivation.

2.3 *In vitro* mastication

In vitro masticatory trials were performed with the Artificial Masticatory Advanced Machine (AM^2) masticator apparatus (**Figure 1**) which was conceived and validated to produce food boluses presenting similar granulometric properties to those measured in boluses collected after *in vivo* mastication. The masticatory disks are of similar contact surface area as the measured between opposite teeth, exerting shear and compression stresses on the food material as those observed during human mastication. A full description of the AM^2 has been provided in previous studies (Mishellany-Dutour et al., 2011; Woda et al., 2010). The programming procedure has already been described in previous work (Peyron et al., 2019; Peyron & Woda, 2016). Briefly, it is classically based on comparisons of *in vivo* vs. *in vitro* particle size distribution (*PSD)* curves and validated when curves of both overlapped.

For this programming step, *in vivo* data came from previous experiments on the same bread product (Traditional French baguette) with 10 young subjects (32 ± 6 years) in good oral health, members of the dental faculty, accustomed in performing masticatory experiments and

bolus expectoration at the moment of swallowing. An average of 25.0 ± 6.4 masticatory cycles in a mean sequence duration of 17 seconds were required to masticate Traditional French baguette samples of 2.6 ± 0.1 g until the swallowing point.

During programming procedure, mean PSD curve of the *in vivo* boluses determined by sieving (see section 2.4) served as the reference curve to be reproduced for *in vitro* bolus granulometry. The amount of saliva incorporated in the *in vivo* bolus during mastication was also implemented during programming of normal *in vitro* masticatory conditions. It was estimated by subtraction of bread sample mass to *in vivo* bolus mass and corresponded to 32.6 ± 3.7 % of bolus mass. During the programming step, milli-Q water was used as oral lubricant, injected at the beginning of the masticatory sequence (mono-injection). The volume of water used (2 mL) was based on the amount of saliva determined by weighting the *in vivo* ready-to-swallow bolus and an estimation of ~1 mL related to the oral coating that saliva would form in mouth (Collins & Dawes, 1987). During the experimental step of the work, the salivary fluid tested was added in the masticatory chamber after the food sample and just at the beginning of the masticatory sequence. The bread bolus was recovered at the end of mastication with a spatula through the opened masticatory chamber and rapidly analysed. In case of verifying the absence of post-mastication enzymatic alteration of the bolus, acid was immediately added to the bolus.

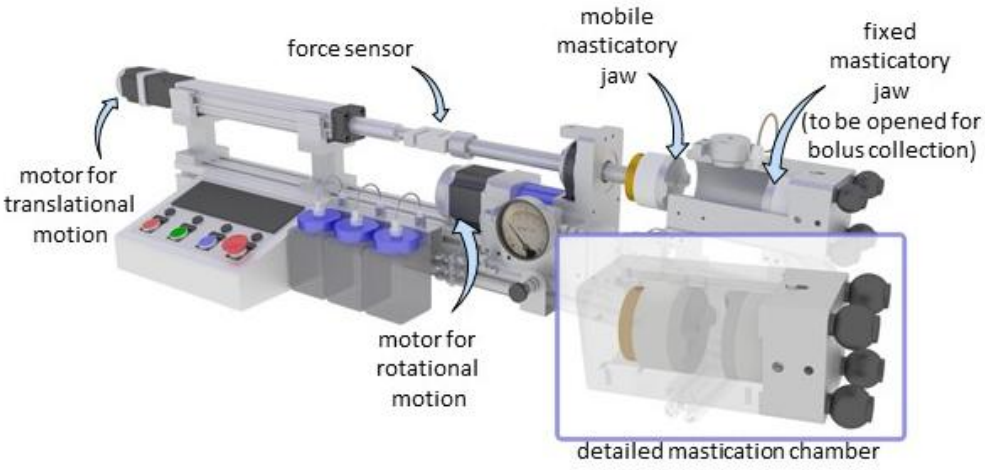

Figure 1. AM^2 artificial masticator. An insert of an open view of the mastication chamber (dimensions: 19.5 × 9.5 cm) is also provided, showing the fixed and the mobile masticatory jaws.

2.4 Particle size distribution in food bolus

Particle size distribution (*PSD*) by granulometric analysis was performed based on dry sieving, providing relevant information on the physical features of bread boluses. Immediately after collection, the food bolus was poured on a 300 µm soft sieve and rinsed with running water. The soft sieve covered with well-spread particles is placed for 30 minutes in a ventilated oven (UFE 400–800, Memmert, Germany) at 37 ± 1°C. The dried bolus was placed on a sieve tray column extractor of nine sieves with different size (7.1, 6.3, 4.0, 2.5, 2.0, 1.5, 1.0, 0.8, 0.4 and < 0.4 mm), being sieving operated with a mechanical sieve shaker operated for 3 min at a vibratory amplitude of 1.7 mm (Retsch Gmbh, AS 200-digit CA, Germany). Particles retained on each sieve were then weighed and expressed as cumulative particle mass passing through each sieve. The d_{50} values (theoretical sieve size through which 50% of the bolus mass can pass) were extracted from the individual cumulative curves by graphical projection on the x-axis. The *PSD* curves and the d_{50} values were used for bolus comparison.

2.6. Statistical analysis

Statistical analysis was performed with SPSS software (IBM SPSS Statistics, V28). To validate the masticator programming, a One-Way repeated-measure ANOVA was performed in a General Linear Model design (with sieves as the repeated within-subjects factor) to confirm no differences between *PSD* of *in vivo* and *in vitro* bread boluses. The Mauchly's test was used to assess whether the assumption of sphericity was met, and the Lower-Bound corrections was applied in case of no sphericity. The bolus mass retained in each sieve was the variable measured, being repeated through the ten sieves. The same kind of One-Way ANOVA was then conducted to compare the *PSD* in boluses produced under the different

conditions investigated (type of salivary fluid, quantity, temperature, and the presence or absence of enzyme). A One-Way ANOVA was also conducted to verify whether significant differences exist between the percentage of saliva calculated for the different boluses or between the d_{50} values. When a difference was observed, the mean comparison was obtained using a Student-Newmann-Keuls test carried out with a risk fixed at 5%.

3. Results

3.1. Validation of AM^2 masticator programming

Figure 2 depicts a comparison between the average particle size distribution (*PSD*) curves of *in vivo* and *in vitro* bread boluses collected at the end of the masticatory sequence. The results indicate that the bread boluses generated by the AM^2 masticator with 25 masticatory cycles and 2 mL of water as an oral lubricant (delivered as a mono-injection at the beginning of the mastication sequence) were not significantly different from *in vivo* boluses in terms of *PSD* ($p=0.131$). This validates the AM^2 programming to generate *in vitro* boluses that resemble the granulometric properties of *in vivo* boluses. However, the mass of *in vivo* boluses was significantly lower than those of the collected *in vitro* ($p<0.001$) (**Figure 3a**), suggesting that *in vivo* a considerable portion of the bolus is swallowed and escapes analysis (~40 mg). Nonetheless, there was no significant difference between the salivary fluid content of *in vivo* and *in vitro* boluses ($p=0.281$; 32.6 ± 3.7 % and 36.1 ± 1.0 %, respectively, as shown in **Figure 3b**). Similarly, there was no significant difference in the d_{50} values, which were 4.1 ± 0.7 mm and 4.5 ± 0.4 mm for *in vivo* and *in vitro* boluses respectively ($p=0.194$; **Figure 3d**). In summary, the AM^2 masticator can produce boluses of Traditional French Baguette with comparable granulometric properties and salivary fluid content to those generated by humans during oral processing of bread, being a reliable tool for further investigation.

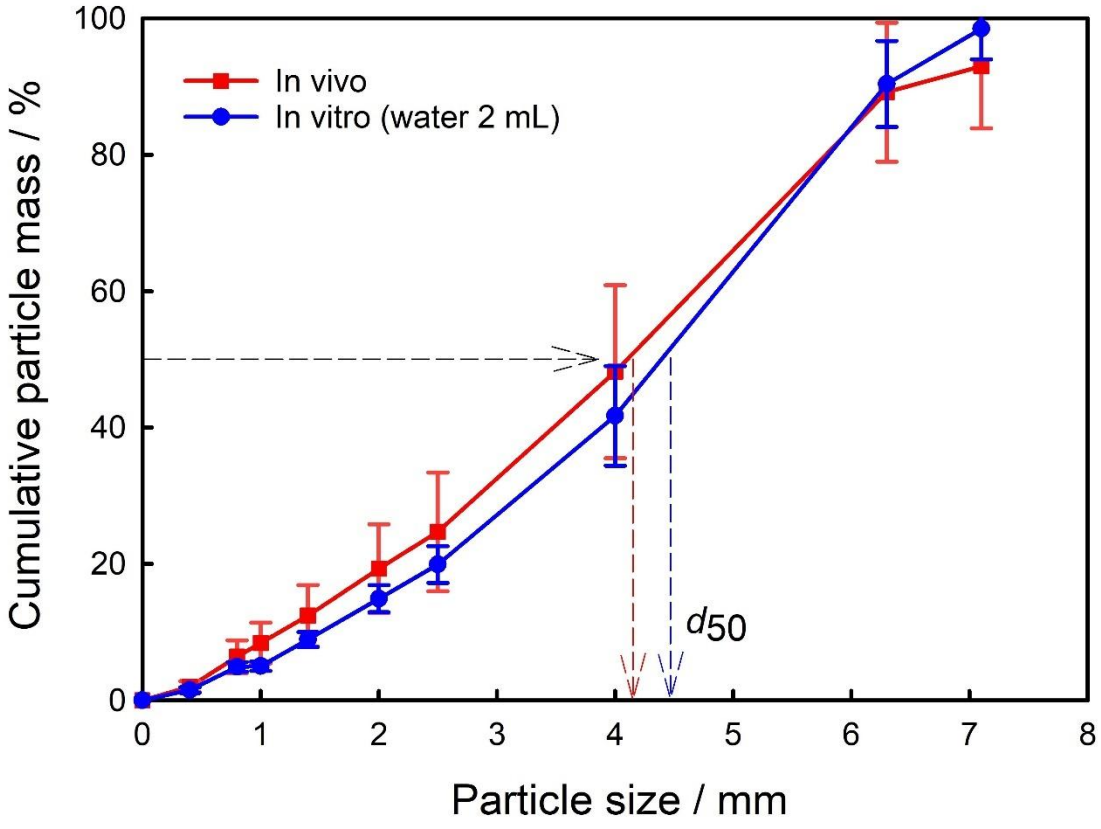

Figure 2. Particle size distribution curves of bread boluses collected at the end of *in vivo* and *in vitro* masticatory sequence (n = 10 for each). Overlapping of these curves helped validating the programming of the AM^2 masticator apparatus. No statistical differences were found between both curves (p=0.131). Mean values are reported along with standard deviations. The method of extracting the median particle size by mass ($d_{50,}$ aperture of the theoretical sieve throughout which the 50% of the bolus particle mass could pass) is also illustrated.

3.2 Effect of the salivary fluid type on *in vitro* bread boluses characteristics

Regardless of whether water or basic saliva fluid (BSF) was utilised for *in vitro* mastication, there were no significant differences observed in either the mass of the bolus or its salivary fluid content (p>0.05; Table 2). The *in vitro* boluses formed using water and BSF weighed 4.1 ± 0.1 g and 4.0 ± 0.3 g, as presented in **Figure 3a**, and exhibited a salivary fluid content of approximately 35% (**Figure 3b**). Moreover, no significant differences were detected in terms of *PSD* or d_{50} values (p > 0.05; Table 2; **Figures 3c** and **3d**), with d_{50} values of 4.5 ± 0.4 mm and 4.1 ± 0.4 mm for those using water or BSF, respectively.

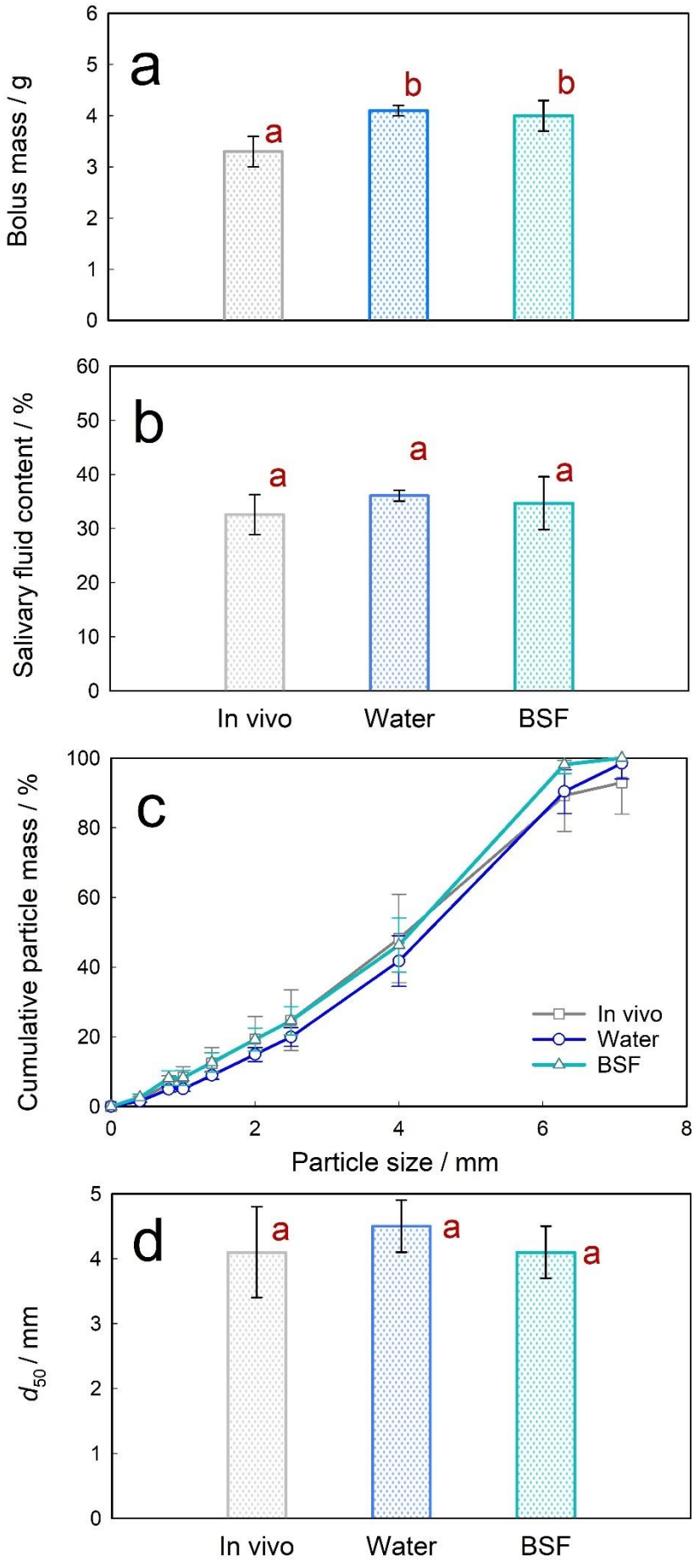
a
Bolus mass / g
6
5
4
3
2
1
0
a
b
b
b
Salivary fluid content / %
60
50
40
30
20
10
0
a
a
a
In vivo
Water
BSF
c
Cumulative particle mass / %
100
80
60
40
20
0
0
2
4
6
8
Particle size / mm
In vivo
Water
BSF
d
d_{50} / mm
5
4
3
2
1
0
a
a
a
In vivo
Water
BSF

Figure 3. Effect of the type of salivary fluid on (a) bolus mass (including incorporated salivary fluid), (b) bolus salivary fluid content, (c) bolus particle size distribution (*PSD*), and (d) median particle size (d_{50}) of the bolus formed during *in vitro* mastication: water or Basic Salivary Fluid (BSF) and compared to *in vivo* data (reference). Mean values (n=10 for each) are reported along with standard deviations. Experimental conditions are reported in Table 1 and statistics in Table 2. All ANOVA procedures were followed by a Student-Newmann-Keuls test and small red letters indicate significant differences (p<0.05) between data groups, considering a complete model with *in vivo*, water and BSF as factor levels.

3.3 Effect of absence of salivary fluid on *in vitro* bread boluses characteristics

The characterisation of *in vitro* bread boluses generated under healthy masticatory conditions without the use of any oral lubricant was also conducted, revealing a final bolus mass that was comparable to the initial sample mass of 2.6g (**Figure 4a**), obviously without presenting any fluid addition in the final bolus (**Figure 4b**). The mean *PSD* curve of these bread boluses in the absence of salivary fluid is depicted in **Figure 4c**. As anticipated, significant differences were observed for *PSD* between *in vitro* boluses prepared either with or without any oral lubricant (p<0.001; Table 2). The bolus resulting from *in vitro* mastication without any fluid was visibly compressed and constituted by hard and very large fragments that did not allow for d_{50} extraction. At the end of the mastication sequence in absence of salivary fluid, nearly 80% of the bolus particles had a size greater than 7.1 mm (**Figure 4c**; Table 2).

3.4. Effect of the salivary fluid quantity on *in vitro* bread bolus characteristics

To mimic normal mastication under conditions of hypersalivation, *in vitro* bread boluses were produced using an excessive amount of saliva (BSF, 4 mL), and subsequently compared to boluses generated under normal salivary conditions (BSF, 2 mL). The amount of saliva used was intended to simulate a salivary flow rate of 10 mL/min during mastication, with an additional 1 mL being estimated for the salivary oral coating. At the end of the complete masticatory sequence, a significant difference in bolus mass was observed (F=8.88, p=0.011; Table 2), with values of 4.0 ± 0.3 g and 5.0 ± 0.9 g being obtained for healthy salivary and hypersalivation-like conditions, respectively (**Figure 4a**). The degree of salivary fluid

incorporation into the *in vitro* bread bolus also significantly differed (F=9.36, p=0.009; **Table 2**), with approximately 10% more salivary fluid being present under conditions of excessive salivation (**Figure 4b**). Additionally, significant differences were observed in the *PSD* curves (F=14.05, p=0.002; **Table 2**) and mean d_{50} values (F=17.83, p<0.001; **Table 2**), revealing d_{50} values of 4.1 ± 0.4 mm and 3.1 ± 0.5 mm for 2 mL and 4 mL, respectively (**Figures 4c and 4d**). These outcomes imply an increased level of bolus fragmentation during mastication, leading hypersalivation to bread boluses composed of much smaller particles than those formed *in vivo* or *in vitro* under normal salivary conditions. In fact, isolated particles above 6 mm were scarcely found.

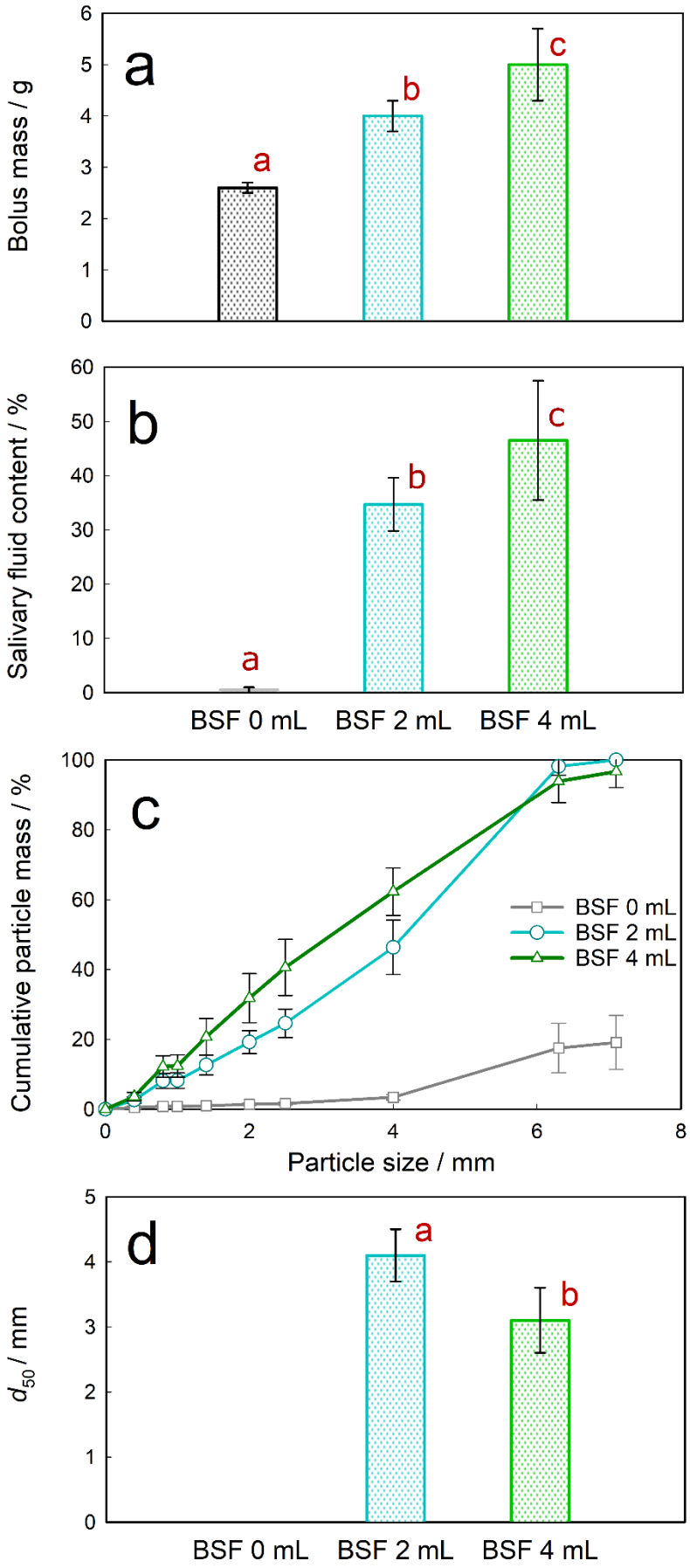
a
Bolus mass / g
0
1
2
3
4
5
6
a
b
c
b
Salivary fluid content / %
0
10
20
30
40
50
60
a
b
c
BSF 0 mL
BSF 2 mL
BSF 4 mL
c
Cumulative particle mass / %
0
20
40
60
80
100
0
2
4
6
8
Particle size / mm
BSF 0 mL
BSF 2 mL
BSF 4 mL
d
d_{50} / mm
0
1
2
3
4
5
a
b
BSF 0 mL
BSF 2 mL
BSF 4 mL

Figure 4. Effect of the Basic Salivary Fluid (BSF) volume (0, 2 or 4 mL, imitating the absence of saliva, normal or hypersalivation) on (a) bolus mass (including incorporated salivary fluid), (b) bolus salivary fluid content, (c) bolus particle size distribution (*PSD*), and (d) median particle size (d_{50}) of the bolus formed during *in vitro* mastication. Mean values (n=10 for BSF used at 2mL, n=5 for BSF used at 0mL and at 4mL) are reported along with standard deviations. In absence of oral lubricant, bolus d_{50} value is above 7.1 mm (maximum sieve size used). Experimental conditions are reported in Table 1 and statistics in Table 2. All ANOVA procedures were followed by a Student-Newmann-Keuls test and small red letters indicate significant differences ($p<0.05$) between data groups, considering a complete model with BSF at 0, 2 and 4mL as factor levels.

3.5. Effect of both salivary fluid temperature and enzymatic action on the *in vitro* bread boluses characteristics

The impact of salivary fluid temperature (20 and 37°C) and the presence of human salivary α-amylase on bread bolus properties were also investigated under normal both salivary volume (2 mL) and mastication (**Figure 5**). Results indicate that BSF temperature did not affect any of the bolus characteristics studied, including bolus mass, fluid content, *PSD* curves, or mean d_{50} values ($p>0.05$; **Table 2**). However, the presence of salivary enzyme at 37°C did. Despite human salivary α-amylase did not impact bolus mass or fluid content (**Figure 5a** and **5b**), it significantly impacted bolus fragmentation ($F=18.02$, $p<0.001$; **Table 2** and **Figure 5c**), leading to smaller d_{50} values of 2.6±0.3 mm compared to 4.1 mm observed previously *in vitro* and *in vivo* ($F=41$, $p<0.001$; **Table 2** and **Figure 5d**).

To determine if there was any post-mastication alteration of bread bolus characteristics during analysis, enzyme deactivation by acid was also performed, but no significant differences ($p>0.05$; **Table 2**) were observed in bolus mass, fluid content, *PSD*, or d_{50} upon deactivation (**Figure 5**).

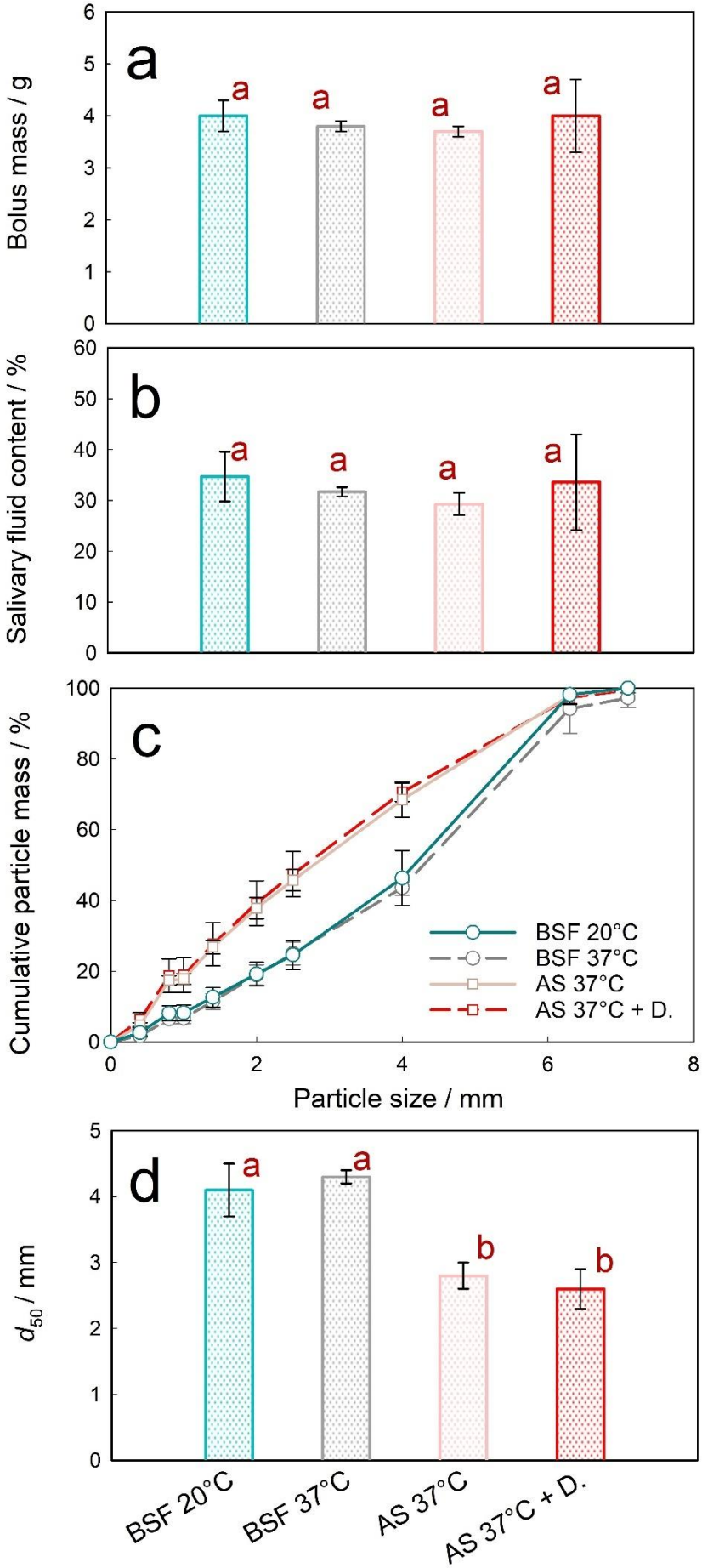
a
Bolus mass / g
0
1
2
3
4
5
6
a
a
a
a
b
Salivary fluid content / %
0
10
20
30
40
50
60
a
a
a
a
c
Cumulative particle mass / %
0
20
40
60
80
100
0
2
4
6
8
Particle size / mm
BSF 20°C
BSF 37°C
AS 37°C
AS 37°C + D.
d
d_{50} / mm
0
1
2
3
4
5
a
a
b
b
BSF 20°C
BSF 37°C
AS 37°C
AS 37°C + D.

Figure 5. Effect of the Basic Salivary Fluid (BSF) temperature, the addition of human salivary α-amylase (AS; 92U/mL) with and without its deactivation (D) using acid (0.01M HCl, pH2) on (a) bolus mass (including incorporated salivary fluid), (b) bolus salivary fluid content, (c) bolus particle size distribution, and (d) median particle size (d_{50}) of the bolus formed during *in vitro* mastication. Mean values (n=10 for BSF at 20°C, n=5 for BSF and AS and AS + D at 37°C) are reported along with standard deviations. Experimental conditions are reported in Table 1 and statistics in Table 2. All ANOVA procedures were followed by a Student-Newmann-Keuls test and small red letters indicate significant differences ($p<0.05$) between data groups.

4. Discussion

The characterisation of both in vivo FOP and food bolus can become challenging due to significant discrepancies in saliva production observed among volunteers, both on an individual level and when comparing different individuals. An excellent solution to address this issue involves employing *in vitro* masticatory systems, which can offer insights into the oral processes contributing to the creation of lifelike food boluses from various unique perspectives, e.g., food fragmentation, hydration of the bolus by saliva, and the process of oral digestion. The AM^2 masticator used in this work was capable of producing *in vitro* boluses with similar particle size distribution and d_{50} values to those produced by healthy individuals during mastication of Traditional French baguette. Another advantage in using this kind of device is that the mass of *in vitro* boluses recovered at the end of the AM^2 masticatory sequence is slightly greater than *in vivo* boluses, due to absence of intermediate swallows that can be frequently observed during *in vivo* oral processing (Hiiemae et al., 1996; Hiraoka et al., 2017).

Extensive research has been conducted on the mastication process of bread and the formation of bread boluses. A range of factors, including bread composition, structure, density, moisture content, crust-to-crumb ratio, and so on, have been identified as major contributors to oral processing. These factors influence various aspects of the process, such as the number of masticatory cycles, muscular forces, tongue activity, and the resulting

properties of the bolus, including its firmness, elasticity, and particle size (Gao et al., 2017; Gao & Zhou, 2021; Le Bleis et al., 2013; Panouillé et al., 2014; Tournier et al., 2012).

Considering the functions of saliva, it has predominantly been portrayed as a pivotal element in the oral processing of food. It plays a role in coating the mouth and food particles, facilitating their aggregation, initiating the hydrolysis of carbohydrates, and providing the necessary rheological conditions for the formation of a swallowable food bolus. During mastication, saliva moistens the particles, resulting in the development of an adhesive force between the particles that leads to the formation of a cohesive bread bolus (Gao et al., 2017; Le Bleis et al., 2013; Tournier et al., 2012). As the masticatory sequence advances, the saliva content progressively increases, causing the bread bolus to become less elastic, less cohesive, and more adhesive, while its softness increases (Gao et al., 2017; Peyron et al., 2019; van Eck et al., 2019). The kinetics of hydration and absorption of saliva can exhibit variations depending on the internal structure of breadcrumbs, as evidenced by research conducted by Mathieu et al. (2016) and Gao et al., (2015). Based on our findings, when considering normal oral conditions, whether in an *in vivo* or *in vitro* setting, the boluses formed from Traditional French baguette boluses revealed that approximately 32-36% of salivary fluid gets incorporated, with an estimated *in vivo* saliva flow rate of 3.4 ± 0.6 mL/min during mastication of this kind of bread (calculated from the ratio of "salivary fluid content/chewing time"), agrees with rates reported for whole saliva stimulated by chewing (Chen, 2009; Heintze, 1984; Sreebny, 2000). In addition to the widely acknowledged attribute of plasticity and rheological behaviour that enhances swallowing through saliva impregnation (Le Bleis et al., 2013), there has been extensive research into the role of salivary alpha-amylase in the biochemical aspects of oral digestion. Despite the well-established and pivotal role of salivary α-amylase in the oral digestion of starchy foods ~~such as~~ like bread, pasta, and rice, which is crucial to the bolus formation (Freitas et al., 2022; Hoebler et al., 1998; Joubert et al., 2017;

Mennah-Govela & Bornhorst, 2016; Ribes, Genot, Aubry, et al., 2023; Zhang et al., 2022), there has been limited research on how it affects the physical properties; any existing studies primarily focused on its rheological or tribological aspects (Laguna et al., 2021). In fact, our study contributes to a deeper understanding of the effect of salivary α-amylase on bread fragmentation by producing a higher proportion of smaller particles with over-disrupted boluses formed in the presence of active enzyme.

Bread, like any other solid and dry food, requires the incorporation of sufficient saliva to achieve hydration and form a bolus that is safe for swallowing. However, in certain clinical scenarios such as ageing, oral pathologies, radiation treatments, salivary gland damage, or polymedication, saliva secretion can be drastically reduced. Until now, the effect of such a reduction or absence of saliva during oral food processing on particle size distribution has not been explored. Our research has demonstrated that the absence of salivary fluid impedes bread fragmentation, resulting in nearly 80% of particles exceeding 7.1 mm in size. This clearly indicates that the bread bolus would not be adequately prepared for swallowing ~~at~~ by the end of the saliva-deprived masticatory sequence. This holds true for both the fragmentation of the bolus and its rheological behaviour, as demonstrated through the analysis of bread boluses masticated within a sealed bag, mirroring normal masticatory conditions (Le Bleis et al., 2013). A decrease in saliva provision may lead to a deficient oral processing of bread, which probably may require greater mastication forces and/or longer processing times to manage the food particles, assemble them and form the bolus. However, both parameters would be limited by the physiological features and oral abilities of each individual, and do not guarantee the formation of a safe bolus that can be swallowed. In clinical practice, the use of salivary substitutes can alleviate the effects of saliva deficiency but their convenience on bolus properties remains largely unknown (Piaton et al., 2021), and additional research in this area would be still required. The absence of saliva could be slightly palliated consuming

certain types of food or drinks, such as carbonated beverages, moisture foods (cherry tomatoes, natural yoghurt, …) or citrus juice, either prior to or concurrently with food intake or to enhance saliva secretion and facilitate swallowing (Gavião et al., 2004). Alternatively, toppings may be employed to assist bolus formation in the absence of saliva (van Eck et al., 2019).

Conversely, when an individual experiences hypersalivation, characterised by an excessive amount of saliva in the oral cavity, bread fragmentation was facilitated, similar to that of the observed previously for rice (Asimi et al., 2022), resulting in over-disrupted bread boluses compared to normal saliva provision. Moreover, the extent of bread fragmentation along with an excessive amount of saliva would also affect other physical parameters, such as bolus consistency, hardness, viscosity, and cohesiveness (Jourdren et al., 2016; Le Bleis et al., 2013, 2016). According to Prinz & Lucas's model, particles coalesce in the food bolus, and an excess of saliva provision during mastication would result in a reduction in bolus coherence, leading to escaped particles and a greater risk of aspiration (Prinz & Lucas, 1997). Nonetheless, individuals with hypersalivation may require shorter mastication times than healthy individuals since an excess of saliva in the oral cavity may elicit partial swallows (Shiozawa & Kohyama, 2011).

Thus, beyond the correlation between bolus hydration by saliva and food properties such as hardness and moisture content (Gao et al., 2017), our findings indicate that the quantity of saliva present in the oral cavity and the enzymatic activity of salivary α-amylase during bread mastication significantly influence the particle size distribution and hydration potential of bread boluses, ultimately determining the physical properties of the bolus and, therefore, potentially impacting the subsequent swallowing process.

Limitations of the study

Akin all *in vitro* experiments, the methodology employed in this study did have certain constraints, such as those related to the properties of saliva and the manner in which it was introduced into the apparatus. Human saliva is evidently not a straightforward solution. Despite being composed of 99% water, it is an intricate blend that exhibits complexity in both its biochemical and physical characteristics, making it challenging to replicate accurately for *in vitro* masticatory experiments (Schipper et al., 2007). Indeed, the distinctive stretching or structural properties of human saliva (Haward et al., 2011; Schipper et al., 2007), or the presence of the thin layer normally coating the mouth (Collins & Dawes, 1987), were not replicated in the laboratory setting, leaving unanswered questions about their potential impact on the masticatory process and bolus characteristics. Nevertheless, when artificial saliva is appropriately prepared, it can mitigate concerns related to instability, certain aspects of its complex physicochemical behaviour, and address several challenges stemming from human variability. Furthermore, accurately measuring human saliva flow throughout the formation of a food bolus is practically impossible due to the influence of numerous uncontrollable factors. Therefore, the chosen experimental approach in this study, which involved a single saliva injection at the initiation of the mastication sequence, represents the most suitable method for examining its influence on the bolus characteristics at the moment of swallowing. This approach allows us to assess this impact independently of any effects that saliva might have on the kinetics of bolus formation. Lastly, despite that in this work the oral digestion time (1.5-2 minutes; considering the total time required for taking all bolus material from the AM^2 masticator before enzyme deactivation) aligns with the timeframe suggested by the INFOGEST network (Brodkorb et al., 2019), it is significantly longer than that reported in *in vivo* experiments (~17 seconds). This may slightly overestimate the effect that enzymes have on *in vivo* bolus particle size. Nevertheless, it is evident that α-amylase role in bread oral

processing is substantial and should be considered for producing realistic *in vitro* bread boluses.

5. Conclusions

In conclusion, besides the established link between bolus hydration through saliva and food bolus attributes such as hardness and moisture content (Gao et al., 2017), our results highlight that both the quantity of saliva in the oral cavity and the enzymatic activity of salivary α-amylase during bread mastication play a substantial role in shaping the size distribution of particles and the hydration potential of bread boluses. The absence of saliva resulted in compromised oral processing of the bread, yielding boluses characterised by excessively large particles, posing potential safety concerns for swallowing. Conversely, an excess of saliva led to excessively disrupted bread boluses, characterised by smaller particle sizes analogous to those affected by human salivary α-amylase. While in real life an excess of saliva may necessitate multiple partial swallows, it is imperative to carefully consider the ramifications of salivary deficiency (also affecting enzyme availability) from a clinical perspective. Such oral dryness in the context of food ingestion could potentially be addressed in the future by the development of dedicated salivary substitutes, although their influence on food oral processing remains unclear. Lastly, this study also emphasizes the importance of replicating realistic salivary conditions in *in vitro* investigations to faithfully recreate the properties of bread boluses.

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Table 1. Experimental conditions. Salivary fluids used during *in vitro* mastication trials, including type, volume, temperature, and the number of repeats performed (n). In some cases, α-amylase has been deactivated after bolus collection using acid (0.01M HCl, pH2). BSF and AS refer to Basic Salivary Fluid and Artificial Saliva containing human salivary α-amylase, respectively.

Type of oral lubricant	**Salivary conditions simulated**	**Fluid volume (mL)**	**Fluid temperature (°C)**	**n**	**Human salivary α-amylase**	
					Concentration (U/mL)	Deactivation
Water	Normal salivation	2	20	10	-	-
BSF	Normal salivation	2	20	10	-	-
BSF	Normal salivation	2	37	5	-	-
-	Absence of saliva	0	-	5	-	-
BSF	Hypersalivation	4	20	5	-	-
AS	Normal salivation	2	37	5	92	No
AS	Normal salivation	2	37	5	92	Yes

Table 2. Statistical analysis results. One-Way ANOVAs (in complete or simple statistical models depending on factor levels considered) were conducted to test whether significant differences existed between the bolus mass, the percentage of saliva incorporated in boluses and d_{50} values obtained in the different conditions (type of salivary fluid, quantity, temperature, and the presence / absence of enzyme and its deactivation). One-Way repeated-measures ANOVAs (in complete or simple statistical models depending on factors considered) were performed in a General Linear Model design to test if no difference was observed between particle size distribution (*PSD)* of *in vivo* and *in vitro* bread boluses obtained in the different conditions (type of salivary fluid, quantity, temperature, and the presence / absence of enzyme and its deactivation). The Mauchly's test was used to assess whether the assumption of sphericity was met, and the Lower-Bound correction was applied in case of no sphericity.

	Factor type	**Factor levels**	**Bolus mass** [1] **(g)**	**Salivary fluid incorporated** [1] **(%)**	d_{50} [1] **(mm)**	***PSD*** [2] **(mm)**
ANOVA complete model	Type of salivary fluid	*In vivo* Water BSF	*F*=25.97 *p*<0.001	*F*=1.35 *P=0.281*	*F*=1.75 *p*=0.194	*F*=3.58 *p*=0.082
	Volume of salivary fluid	0mL 2mL 4mL	*F*=26.71 *p*<0.001	*F*=60.49 *p*<0.001	*F*=493 *p*<0.001	*F*=134.9 *p*<0.001
	Type and/or temperature of salivary fluid	BSF 20°C BSF 37°C AS 37°C AS + D 37°C	*F*=0.61 *p*=0.615	*F*=0.58 *p*=0.639	*F*=41 *p*<0.001	*F*=18.02 *p*<0.001
ANOVA simple model	Type of salivary fluid	Water BSF	*F*=0.27 *p*=0.61	*F*=0.34 *p*=0.566	*F*=3.57 *p*=0.075	*F*=2.3 *p*=0.146
	Volume of salivary fluid	2 mL 4 mL	*F*=8.88 *p*=0.011	*F*=9.36 *p*=0.009	*F*=17.83 *p*=0.0009	*F*=14.05 *p*=0.002
	Temperature of salivary fluid	BSF 20°C BSF 37°C	*F*=1.52 *p*=0.241	*F*=1.67 *p*=0.22	*F*=0.79 *p*=0.39	*F*=1.37 *p*=0.257
	Type of salivary fluid	AS 37°C AS 37°C + D	*F*=0.58 *p*=0.472	*F*=0.52 *p*=0.495	*F*=1.12 *p*=0.326	*F*=0.24 *p*=0.642

BSF: Basic Salivary Fluid; AS: Artificial Saliva containing amylase; D: deactivation of amylase with acid

[1] *One-Way ANOVAs*
[2] *Repeated-Measures ANOVAs*