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Comparison of Gelatin Flavors for Oral Dosing of C57BL/6J and FVB/N Mice

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Precise oral dosing in rodents is usually achieved by intragastric gavage. If performed incorrectly due to technical difficulties, inexperience, or animal resistance, oral gavage may have animal welfare implications such as esophageal and gastric rupture and aspiration. The stress that is induced by this procedure can also lead to confounding results. In several animal models, drug vehicles must be sugar-free, deliver drugs in a specific formulation, and sometimes supply water. Gelatin has all of these properties. The current study aimed to evaluate the use of gelatin vehicles with different sensory features as an alternative to oral gavage. We investigated the time taken by 2 different inbred mouse strains, FVB/N and C57BL/6J, to ingest sugar-free gelatin pellets of varying flavors. Results showed that FVB/N mice took more time to eat the unflavored, strawberry and diet-flavored gelatin pellets than did C57BL/6J mice. Both strains showed low preference for lemon flavor, with the same ingestion times after the second day. This study showed that the C57BL/6J mice are more likely to eat gelatin than are FVB/N mice, and that the 2 strains of mice show a lower preference for lemon flavoring as compared with other flavors. This method of voluntarily oral administration offers an alternative to gavage for studies that use oral dosing studies.

Abbreviation: BF, broccoli flour

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Drugs or other substances can be administered to laboratory rodents by several routes. When designing a study that includes drug administration, the optimal delivery route and animal welfare should be considered, especially when the substance has to be administered repeatedly for a long period. The refinement of procedures is an important ethical issue in an experimental protocol and helps to promote reliable results. When the animal is experiencing pain, discomfort, or even stress resulting from routine handling, the body temperature, blood pressure, heart rate, corticosterone, prolactin, and glucose levels increase,[3,7,17,36] and the behavior of the animal is also altered.[3] These changes may have animal welfare implications and can compromise the experimental results.

When administering substances to laboratory rodents, compounds can be incorporated into the diet or drinking water. However, animals may not ingest the required individual dose, or the test compound may not be suitable for incorporation in the food or water due to its chemical stability or solubility. For these reasons, oral administration is mostly done by oral gavage,[14] which is fast and allows the delivery of the correct dose directly into the stomach. Nevertheless, this technique requires a trained and proficient technician due to the risks it presents to the animal`s welfare. One study[7] showed that oral gavage increased the plasma levels of corticosterone in rats and that lipid vehicles delivered by gavage induced a similar response in a volume-dependent manner. Another study demonstrated that oral gavage increased fecal corticosterone metabolites and altered the blood pressure and heart rate for 3 to 5 h in mice.[36] The stress induced by the restraint and introduction of the gavage needle[6,21,29] can be reduced by precoating the gavage needles with sucrose.[16] However, other serious physical injuries such as gastric distention, aspiration pneumonia, esophageal and gastric rupture, and even death may occur.[1,3,28,34] Therefore, refined methods of oral dosing and alternatives to oral gavage would be useful. Oral administration through voluntary ingestion of a gelatin vehicle was reported for glucose administration to mice during oral glucose tolerance test[8] and for the administration of a cannabinoid-1 receptor antagonist in mice.[37] In rats, the voluntary ingestion of a buprenorphine jelly was tested for postsurgical analgesia[11] and palatable gelatin tablets were tested for delivery of the wake-promoting drug modafinil.[9] However, none of these studies evaluated how long the animals took to ingest gelatin and how the sensory characteristics of the vehicles influences intake duration. The time that the animal takes to eat the entire gelatin pellet must be standardized because variation may alter the onset of effect and influence pharmacokinetic measures. The current study evaluated 2 different mouse strains, C57BL/6J and FVB/N, with regard to the acceptance of and time taken to consume a whole gelatin pellet of 4 different flavors (unflavored, strawberry, lemon, and diet-flavored); we subsequently used this methodology to test voluntary ingestion of broccoli flour.

Materials and Methods

Ethics statement. This study was conducted in accordance with Portuguese Law (DL n°113/2003) and the European Directive 2010/63/EU on the protection of animals used for scientific purposes. All of the animal experiments were approved by the Animal Welfare and Ethical Review Body of University of Trás-os-Montes and Alto Douro (UTAD) and by the national

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Animals. Male 6-wk-old SPF C57BL/6J mice (n = 35) were purchased from Charles River Laboratories (France) and were housed at the animal facility of UTAD in a conventional room. Mice were used in the study after a week of acclimatization and weighed 20.7 ± 1.7 g at 7-wk-old. Male 8-wk-old FVB/N mice (n = 35) with a conventional health status, weighing 27.2 ± 3.0 g, were obtained from the local colony of UTAD's animal facilities. Mice were housed in groups of 7 in open polycarbonate 1284L Eurostandard Type II L cages (Tecniplast, Italy) with corncob bedding (CORNCOB ULTRA 12, Ultragene, Portugal) and environmental enrichment (cardboard rolls and paper). Cages were changed weekly, coinciding with the beginning and the end of the study. The cage was changed after the trial. All mice had ad libitum access to rodent standard diet (Mucedola 4RF21A Certificate, Milan, Italy) and tap water. Mice were housed under controlled temperature (21 ± 2 °C), relative humidity (50 ± 10%) and 12 h light (0700 to 1900)/12 h dark (1900 to 0700) cycle.

Experimental design. For each strain, mice were assigned into 5 groups (n = 7/group) by simple randomization using Excel, and each group was allocated to a single cage: Group 1, control; Group 2, unflavored gelatin; Group 3, strawberry flavored gelatin; Group 4, lemon flavored gelatin; and Group 5, diet-flavored gelatin. Group 1 was not given gelatin and was used only as a control of body weight. Before the first day of contact with gelatin pellets, mice were fasted for 12 h to encourage them to overcome neophobia.[20] The group 1 was also fasted. Mice were then placed individually in polycarbonate cages without bedding, food, or water, and 1 pellet of gelatin was placed in each cage. When mice were handled, they were gently picked up by the base of the tail with a hand positioned to support the body. The total time that each mouse used to ingest the entire pellet was recorded with a chronometer up to a maximum of 60 min. The mice were returned to their respective cages as soon as they finished eating. No prior training or adaptation was used for these cage changes. On the following days, for a total of 8 d, and at the same time each morning (1000), the same procedures were repeated but the mice were not fasted. The researcher was aware of the treatment of each mouse because the gelatin pellets had different colors. During the experimental trial, the clinical condition of the mice was observed daily. The body weight and the time to ingest the entire pellet were recorded daily for each mouse. The relative weight gain and the average daily food and water intake per mouse were calculated as described below. The food and water consumption calculations are estimates, as spillage was not taken into account.

$$\text{Relative weight gain} = \frac{(\text{Final weight} - \text{Initial weight})}{\text{Final weight}}$$

$$\text{Average daily food intake per mouse } (\text{g}) =$$

$$= \frac{(\textit{Initial weight} - \textit{Final weight})}{(n^o \textit{ days} \times n^o \textit{ animals per cage})}$$

$$\text{Average daily water intake per mouse } (\text{mL}) =$$

$$= \frac{(\textit{Initial volume} - \textit{Final volume})}{(n^o \textit{ days} \times n^o \textit{ animals per cage})}$$

Gelatin preparation. Commercial sugar-free gelatin with different flavors (unflavored (Globo, Portugal), strawberry (Auchan, Portugal) and lemon (Auchan, Portugal)) were purchased from a local market. To have a representation of a sweet aroma and a citrusy aroma, the strawberry and lemon flavors were chosen. In addition to being quite distinct flavors from each other and from the other flavors, they are also easily found on the market. Furthermore, strawberry and lemon flavors have been used in mice and rat studies, respectively. The unflavored gelatin was unsweetened, while strawberry and lemon gelatins contained aspartame and acesulfame-K as sweeteners. The gelatins were prepared according to package instructions, but with half the recommended volume of water. A 96-multiwell plate was used as a mold for the gelatin pellets; 150 µL of gelatin solution was placed in each well. The diet-flavored gelatin was prepared by mixing 1.5 g of standard ground diet with 15 mL of unflavored gelatin. Gelatin pellets weighed about 0.15 g and were stored at –20 °C until use. The day before use, the pellets were placed at 4 °C.

Ingestion of broccoli flour in gelatin pellets. The oral intake of broccoli leaf flour (BF) in gelatin pellets was tested. BF was obtained from lyophilized leaves that were ground into a fine powder. Pellets were prepared with 10 different concentrations: 0.2 g/kg, 0.6 g/kg, 0.8 g/kg, 1.2 g/kg, 1.5 g/kg, 2 g/kg, 4 g/kg, 10 g/kg, 20 g/kg and 40 g/kg to evaluate the ingestion time for each dose. The ingestion time for doses above 1.2 g/kg were very prolonged. The lower doses (0.2 g/kg to 0.8 g/kg) were evaluated in unflavored gelatin, prepared as described above. These unflavored gelatins were either unsweetened or were sweetened with the addition of 10% sucrose in order to mask any some unpleasant taste. The average body weight of each mouse was used to prepare the gelatin pellets with the correct dose for each mouse. The BF necessary for 16 mice was mixed with 5 mL of sweetened or unsweetened gelatin solution in round cups. Gelatins were placed at –20 °C for a few hours to facilitate the precise cutting of the large round gelatin pellet into 16 equal parts, which were then stored at 4 °C until use within 72 h. Pellets weighed around 0.381 ± 0.004 g.

The BF study used 105 8-wk-old, male C57BL/6J mice weighing 23.0 ± 0.4 g (Charles River Laboratories, France). Mice were housed at UTAD animal facilities and were used after 2 wk of acclimatization. Mice were fasted overnight. In the morning, mice were placed in individual cages to assess the time taken to ingest each pellet.

Statistical analysis. The data are expressed as mean ± SEM, and n represents the number of independent subjects. Statistical analysis was carried out using GraphPad Prism 7 software. To determine whether data followed a Gaussian distribution, data were analyzed for normality using Shapiro–Wilk normality test. Weight gain differences were evaluated using *one-way* ANOVA followed by Tukey Test or Bonferroni test as specified in the legend. Differences in time required for gelatin ingestion between the 2 strains at each time point were assessed using nonparametric Mann–Whitney *U*-test. Values of $P < 0.05$ were considered statistically significant.

Results

General findings. Average daily food and water intake per mouse for each gelatin flavor during the study is shown in Table 1). Descriptive values were similar between strains and groups, except for the lemon group of C57BL/6J mice, which appeared to have a lower food intake than did the other groups.

The mice did not show any changes in general appearance or behavior during the experiment. The body weight at the

Table 1. Average daily food (g) and water (ml) intake per mouse during the study.

	C57BL/6J		FVB/N	
Group	Food	Water	Food	Water
Control	4.9	4.8	4.2	4.1
Unflavored	3.7	4.6	3.8	4.1
Strawberry	3.6	3.9	3.8	4.6
Lemon	2.8	4.1	4.2	4.9
Diet-flavored	3.8	4.3	3.8	4.7

C57BL/6J and FVB/N mice were given pellets of gelatin with different flavors: unflavored, strawberry, lemon or diet-flavored, for 8 d. Mice were fasted during 12 h before the first day of contact with gelatin pellets. Food and water were weighed at the beginning and at the end of the study. The value of consumption was then divided by the number of days and the number of mice per cage to estimate the average consumption per mouse per day.

beginning and end of the study and relative weight gain are shown in Table 2). In all groups, the initial body weight of C57BL/6J mice was substantially lower than that of FVB/N mice; however, the relative weight gain of C57BL/6J mice was significantly higher ($P < 0.05$) than that of FVB/N mice (Table 2) when compared with the same group. With regard to the relative weight gain values between groups of C57BL/6J mice (Table 2), only the strawberry group registered a significantly lower relative weight gain ($P < 0.05$) as compared with the other groups. Regarding FVB/N strain (Table 2), only the control group recorded a positive relative weight gain, while the lemon group registered the highest weight loss ($P < 0.05$) as compared with the other groups. For both strains, the weight loss was similar after the fasting period (Table 2); however, the C57BL/6J mice regained the initial body weight faster than did the FVB/N mice.

Voluntary intake of gelatin. For each day of the study, the percentage of C57BL/6J and FVB/N mice that ate the whole gelatin pellet within 60 min is shown in Table 3). All C57BL/6J mice ate the whole gelatin pellet in less than 60 min, except for the lemon group, whose percentage ranged from 57 to 86% (Table 3*)*. In FVB/N strain, the percentage of mice that ingested the gelatin pellet within 60 min was very variable between groups (Table 3). The percentage of the unflavored group fluctuated between 14 and 100%, the strawberry group was between 43 and 100%, and the lemon group was between 29 and 100%. The diet-flavored group was between 86 and 100%, except at day 2, on which only 43% of mice ingested the gelatin pellet within 60 min.

The time that each mouse took to eat the whole gelatin pellet on each day of the experiment is shown in Figure 1). Comparing the ingestion times of FVB/N and C57BL/6J mice for each flavor tested, the FVB/N mice took significantly longer for unflavored ($P < 0.05$; Figure 1 A), strawberry ($P < 0.05$; Figure 1 B) and diet-flavored groups ($P < 0.05$; Figure 1 D). For lemon group, the differences were statistically different between the 2 strains only on day 1 and 2 ($P < 0.05$) (Figure 1 C). C57BL/6J mice ingested the unflavored, strawberry and diet-flavored gelatin pellets within 3 min on each study day (Figure 1 A, 1 B and 1 D, respectively). However, the average time to consume the lemon pellet was higher and varied between 13 and 30 min during the experiment (Figure 1 C). All groups of FVB/N mice showed highly variable ingestion times throughout the study (Figure 1), with no preference among the flavors. However, on the 2 last days of the study, the strawberry, lemon, and diet-flavored groups showed more rapid consumption, while the unflavored group was even slower; however, significant differences ($P < 0.05$) were detected only in last day.

Ingestion of broccoli flour in gelatin pellets. Broccoli flour (BF) is very light, and a few milligrams occupy a large volume. As a result, the highest doses (20 g/kg and 40 g/kg) took up such a large volume that preparing a gelatin pellet was not possible and resulted in a large bolus aggregated by the gelatin solution that could not be eaten by a mouse in a short time. The medium doses (1.2 g/kg to 10 g/kg) were also too large to be ingested in a short time. As a result, only the lower doses (0.2 g/kg to 0.8 g/kg) were used. Table 4) shows the average time that C57BL/6J mice took to eat a whole gelatin pellet containing BF. The highest doses tested, 0.6 and 0.8 g/kg BF, were eaten only when sweetened, while for the lowest dose, 0.2 g/kg BF, ingestion times were similar between sweetened and unsweetened. For the doses 0.6 g/kg BF and 0.8 g/kg BF, the fasted mice could eat a pellet that weighed around 0.381 ± 0.004 g in less than 10 min. However, that occurred only with the sweetened pellets.

Discussion

Our study showed that C57BL/6J mice ate the unflavored, strawberry and diet-flavored gelatin pellets faster than did FVB/N mice. The C57BL/6J mice on average took 3 min to eat the entire pellet; thus, this method can be used for this strain. However, both mouse strains showed little interest in lemon flavor. This study also showed a practical example for BF oral dosing by incorporation into gelatin pellets. High doses may require incorporation into sweetened gelatin to mask unpleasant tastes. With this approach, the pellets are ingested more quickly, which is important in bioavailability studies. Furthermore, the physical and chemical properties of the substance may limit the doses that can be administered by using gelatin pellets.

Intragastric gavage is the route of choice for oral administration; however, this technique should be performed by skilled and trained personnel because it can negatively impact animals' welfare. Furthermore, this is a stressful procedure, particularly when performed repeatedly. To minimize adverse effects of oral gavage, the technique can be refined by using a soft teflon probe or gavage needles precoated with sucrose. One group[29] compared the stress response in Wistar rats induced by intragastric gavage with steel and teflon probes and found that the gavage with a stainless-steel probe induced larger alterations in blood pressure and heart rate. In C57BL/6J mice, precoating stainless-steel gavage needles with sucrose decreased the passage time of the gavage needle, reducing the time needed to restrain the mouse and perform the gavage.[16] Moreover, these mice presented less stressful reactions and lower levels of plasma corticosterone than those gavaged with water-coated needles.[16] However, even with these refinements, gavage is still invasive and stressful. Although intragastric gavage is the most common technique used for oral administration and may be necessary in some studies for precise oral dosing, less invasive and stressful alternatives should be used whenever possible. One group[35] also suggests that gavage should be avoided for toxicity testing of endocrine disruptors because gavage does not appropriately model human dietary exposures, by-passes interactions with the oral mucosa, and causes stress that may alter endocrine responses. Although many procedures can cause distress, some are more stressful than others. Voluntary ingestion should induce less stress than the physical restraint associated with forced administration such as gavage.

In the present work, we investigated whether gelatin pellets could be used for voluntary oral dosing in 2 inbred mouse strains, C57BL/6J and FVB/N, and whether animals showed a preference for flavored gelatin. We found that C57BL/6J mice more readily eat gelatin pellets than do FVB/N mice and

Table 2. Body weight (g) at the beginning, after fasting, and end of the study, and the relative weight gain.

	C57BL/6J				FVB/N			
Group	Initial	After fasting	Final	Relative weight gain	Initial	After fasting	Final	Relative weight gain
Control	21.6 ± 0.5	19.4 ± 0.5	22.8 ± 0.7	0.053 ± 0.014[a]	27.4 ± 0.9	25.0 ± 0.9	27.5 ± 0.6	0.004 ± 0.011
Unflavored	19.8 ± 0.3	17.6 ± 0.3	20.9 ± 0.4	0.053 ± 0.005[a]	26.0 ± 0.7	23.7 ± 0.6	24.6 ± 0.6	−0.053 ± 0.009[c]
Strawberry	20.9 ± 0.4	18.3 ± 0.3	21.2 ± 0.4	0.014 ± 0.008[a, b]	28.3 ± 1.1	25.4 ± 1.0	26.1 ± 1.1	−0.083 ± 0.011[c]
Lemon	20.5 ± 1.2	17.9 ± 0.9	21.6 ± 1.2	0.052 ± 0.009[a]	26.3 ± 1.2	23.1 ± 1.3	23.4 ± 1.1	−0.126 ± 0.019[d]
Diet-flavored	20.9 ± 0.6	18.6 ± 0.5	22.5 ± 0.6	0.070 ± 0.007[a]	28.1 ± 1.7	25.7 ± 1.5	26.7 ± 1.4	−0.049 ± 0.014

C57BL/6J and FVB/N mice were given pellets of gelatin with different flavors: unflavored, strawberry, lemon or diet-flavored, for 8 d. Mice were fasted during 12 h before the first day of contact with gelatin pellets. Relative weight gain values are calculated from the initial and final body weight, divided by the final weight.
Data are presented as mean ± SEM (n = 7).
[a]$P < 0.05$, significantly different from FVB/N group in the same row, according to Bonferroni test.
[b]$P < 0.05$, significantly different from the other groups in the same column, according to Tukey test.
[c]$P < 0.05$, significantly different from control group in the same column, according to Tukey test.
[d]$P < 0.05$, significantly different from control, unflavored and diet-flavored groups in the same column, according to Tukey test.

Table 3. Percentage (%) of C57BL/6J and FVB/N mice finishing before 60 min for each day of the study.

		Days							
Group	**Strain**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Unflavored	C57BL/6J	100%	100%	100%	100%	100%	100%	100%	100%
	FVB/N	100%	14%	71%	57%	100%	100%	43%	14%
Strawberry	C57BL/6J	100%	100%	100%	100%	100%	100%	100%	100%
	FVB/N	86%	71%	43%	57%	86%	86%	100%	86%
Lemon	C57BL/6J	86%	86%	57%	71%	71%	71%	71%	71%
	FVB/N	43%	29%	71%	43%	86%	57%	100%	100%
Diet-flavored	C57BL/6J	100%	100%	100%	100%	100%	100%	100%	100%
	FVB/N	100%	43%	100%	86%	86%	86%	100%	100%

C57BL/6J (n = 7) and FVB/N mice (n = 7) were given pellets of gelatin with different flavors: unflavored, strawberry, lemon or diet-flavored, for 8 d. The total time required for the ingestion of the whole pellets was recorded until a maximum of 60 min. Data are expressed as percentage (%) of mice that finished ingestion before 60 min for each day of the study.

that lemon flavor is not appealing. In our protocol, mice were temporarily moved from the home that contained bedding, environmental enrichment, and group housing to a new empty cage without cage mates. Mice were returned to their original cage within an average of 7 min for C57BL/6J mice and 30 min for FVB/N mice. The presence of bedding can interfere with gelatin ingestion for 2 reasons: the probability of the gelatin being contaminated with the bedding and difficulty for the mouse to find it. Using a plate to place the gelatin inside the cage may be an alternative, but the gelatin may be dropped on the bedding and the mouse may take a long time to find it. Our protocol used no prior training or adaptation to these cage changes, which could prolong the time it takes the animal to eat the gelatin due to anxiety or time spent exploring the new cage. In addition, mice were handled by the traditional method in which they were gently picked up by the base of tail with a hand positioned to support the animal's body; this method induces more anxiety-like behaviors than does the cup handling method using 2 hands.[12] However, all mice were subjected to the same handling, and yet some mice ate very fast, while others ate slowly. The fact that C57BL/6J mice ate unflavored, strawberry and diet-flavored gelatin within a few minutes even on day 1 suggests that the lack of adaptation does not interfere with the results or have problematic effects in this mouse strain, and that the longer time to eat the lemon gelatin was due to the low palatability of the flavor. However, such effects cannot be ruled out in relation to FVB/N mice, as they always ate the gelatin relatively slowly, regardless of the flavor. Thus, possible interference could potentially be reduced by giving the mice a training period in which they could become familiar with the new cage before introducing the gelatin pellet.

The initial body weight of C57BL/6J mice was significantly lower than that of FVB/N mice. This was expected because the C57BL/6J mice were younger, and this strain is also naturally lighter than FVB/N.[22,23] Both C57BL/6J and FVB/N mice lost weight after the fasting period, an average of 2.37 g and 2.62 g, respectively; this is a normal physiologic consequence of fasting, because mice consume around two-thirds of their daily food and water intake during the dark phase.[18] C57BL/6J mice regained their initial body weight faster than the FVB/N mice. In fact, FVB/N mice had not recovered their initial weight after one week, except for the control group. Because food and water intakes were similar between the 2 mice strains during the study, the relative weight gain may not be correlated with food and water intakes, especially food intake. As mice age, weight gain is naturally slower; however, the age difference between the strains may not be enough to justify the observed differences. Moreover, mice of the same strain but in different groups also showed differences in weight gain.

The idea of using gelatin vehicles to ingest voluntarily oral drugs to avoid intragastric gavage-induced stress in laboratory animals is not new. Oral delivery of buprenorphine incorporated in raspberry flavored gelatin, given one hour before the induction of anesthesia in Wistar rats for a flank laparotomy, was tested 2 decades ago.[11] This method allows repeated dosing of the rat undergoing surgery without causing additional stress or pain due to restraint for an invasive administration route. To avoid the stress induced by gavage, another group[37]

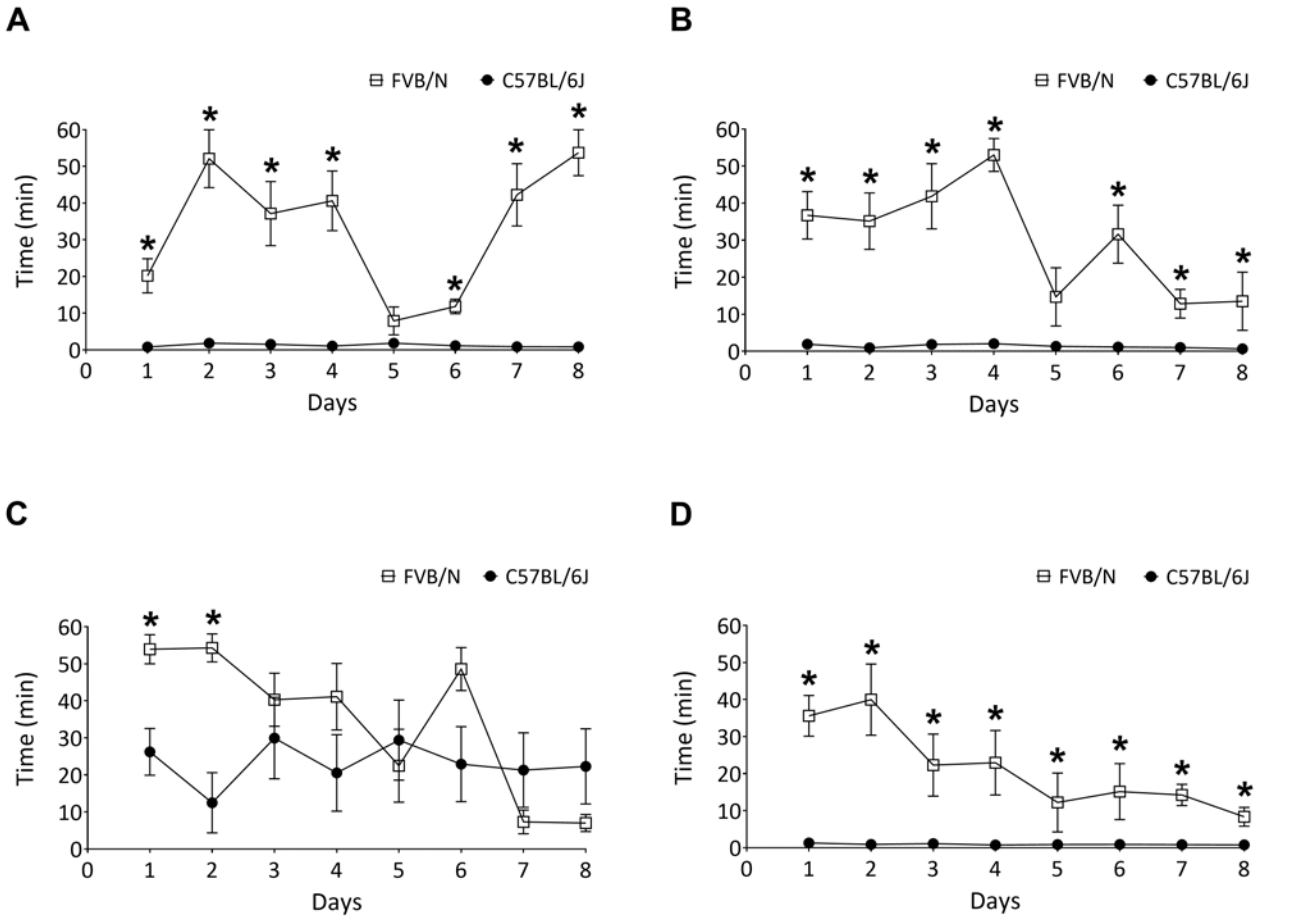

Figure 1. Time of ingestion of gelatin pellets in C57BL/6J and FVB/N mice. Mice were given pellets of gelatin with different flavors: (A) unflavored, B) strawberry, C) lemon or D) diet-flavored, for 8 d. The total time required for the ingestion of the whole pellet was recorded for a maximum of 60 min. The time of 60 min was registered when an animal did not eat the entire pellet. Data are expressed as mean ± SEM ($n = 7$). *$P < 0.05$, significantly different as compared with C57BL/6J mice at the same time point. Nonparametric Mann–Whitney test.

incorporated rimonabant into strawberry-flavored and sweetened gelatin for voluntary oral administration to neuropeptide Y knockout mice (C57BL/6 to 129/SvJ background). Similarly, to overcome the masking of physiologic responses to gut hormones caused by stress-induced oral gavage, another study[8] performed the oral glucose tolerance test in wild-type and peptide YY knockout mice (C57BL/6 to 129/SvJ background) by incorporating glucose into strawberry-flavored gelatin for voluntary ingestion. Another group[9] demonstrated that palatable gelatin tablets could be used as a less stressful oral delivery technique in rats. Rats were single-housed and given gelatin tablets of sugar-free fruit juice concentrate containing modafinil. These gelatin tablets were documented to be effective in delivering modafinil because the drug presented a similar pattern of brain tissue concentration as was achieved by oral gavage.[9] In all of these studies, the animals were pretrained.

In addition to gelatin, other vehicles have been tested, such as a mixture of 5% or 10% sucrose solution with the respective drug, which was then offered to rats for voluntary consumption using a syringe-feeding technique.[2,31] The voluntary ingestion of a drug emulsified in honey and fed to mice using a ball-tip feeding needle has also been tested.[21] Although the ingestion was voluntary, the mice had to be removed from their cages and be held in one hand without additional restraint while feeding; nevertheless, mice did not avoid contact and require restraint such as that needed for gavage.[21] The addition of honey did not interfere on drug absorption, and the uptake and metabolism of the tested drug was identical by either gavage or voluntary intake of honey emulsion.[21] The mixture of a drug with chocolate for voluntary oral ingestion was also tested in rats.[13] However, some substances present in chocolate such as theobromine and caffeine have moderate toxicity in rodents,[32] what makes this vehicle unsuitable. In another study,[8] mice were successfully given a drug masked in a sweetened, strawberry-flavored paste to mice, whereas others[33] found that strawberry jam could be used for drug administration by voluntary intake in male and female C57BL/6 mice; mice were reported to ingest the jam in less than 5 min, with latency

Table 4. Time (min) taken to ingest broccoli leaf flour (BF) gelatin pellets by C57BL/6J mice.

Doses					
0.2 g/kg BF		0.6 g/kg BF		0.8 g/kg BF	
Unsweet (n = 5)	Sweet (n = 20)	Unsweet (n = 5)	Sweet (n = 20)	Unsweet (n = 5)	Sweet (n = 20)
5.4 ± 0.4	3.1 ± 0.2	a	6.1 ± 0.9	a	5.0 ± 0.6

C57BL/6J mice were given sweetened or unsweetened gelatin pellets with broccoli leaf flour (BF) incorporated with different doses: 0.2, 0.6 or 0.8 g/kg BF. Mice were overnight fasted before the first contact with gelatin pellets. The total time required for the ingestion of the entire pellet was recorded.
Data are expressed as mean ± SEM.
[a]After 30 min, not all animals had eaten.

times below 1 min.[33] The sweetness of the vehicle undoubtedly favors the success of these alternative methods,[26] especially when a unpalatable potentially bitter taste of the drug could be an obstacle for the voluntarily oral delivery. Still, despite the sweet flavor, a conditioned taste aversion may occur if the drug in question causes adverse side effects.[2] Alternative flavors other than sweet can be used. One group[36] evaluated a pill formulation in a bacon-flavored soft diet (marketed as Transgenic Dough Diet) that was consumed voluntarily by mice. C57BL/6 mice ate the entire pill within 30 min after it was placed in the cage,[36] which is considerably slower than consumption a sweet vehicle.[9,21,37] That study assessed the heart rate, blood pressure and fecal corticosterone metabolites after voluntary ingestion of the pill or after gavage. Unlike gavage, pill consumption did not induce any significantly stress responses.[36] In all these studies, the animals were pretrained.

Differences in the pharmacokinetics of compounds given by intragastric gavage and oral administration should be considered when designing a study. The intragastric gavage technique consists of inserting a tube into the oral cavity and sliding it through the esophagus to the stomach.[35] Substances are delivered directly to the stomach, allowing accurate and precise dosage and time of administration. Substances delivered by this method are absorbed from the gut and then transported directly to the liver via the mesenteric vessels.[35] This first-pass metabolism results in a lower bioavailability of the compounds.[15] Oral administration using an edible vehicle allows the compound to interact with the buccal, sublingual, palatal, gingival, and labial mucosa[30] before reaching the stomach. At the oral cavity, this interaction can lead to some absorption due to the rich vascular supply of the mucosa and the lack of a stratum corneum epidermis.[30] Substances can be quickly absorbed in this way, avoiding first-pass metabolism and resulting in a higher bioavailability.[35] Thus, oral administration may involve both oral transmucosal and gut absorption. Voluntary oral administration through an edible vehicle could fail with regard to the accuracy and precision of the dose and the time it is ingested. Despite these differences, some studies show little or no differences between the absorption of compounds by oral administration and intragastric gavage.[9,21]

Some studies or models, such as for obesity, may preclude the addition of sugars, so we tested 4 different flavors of sugar-free gelatin: unflavored, strawberry, lemon and diet-flavored. C57BL/6J mice were quick to ingest all the gelatin pellets except for the lemon; not all mice ate the entire gelatin pellet within the 60 min the course of the study. Although rats consume the lemon flavor in conditioned flavor tests,[4,24,25] our data indicate that this flavor is not highly palatable for mice. In contrast to C57BL/6J mice, the FVB/N mice showed variation of ingestion of all the flavors tested. These results suggest that when considering this alternative method, the flavor and the mouse strain should be carefully chosen with regard to strain-specific preference for certain flavors that will promote the intake of gelatin vehicles in a short time. Murine genetic variance strongly affects not only food and water intake in general, but also influences the intake of saccharin, sugars, salts, bitter substances, glutamate and umami-type flavors, fats and ethanol.[5] Genetic differences may have been responsible for the intra- and interstrain variation observed for different flavors in our study.

Although voluntarily oral delivery can substitute intragastric gavage, the voluntary approach also has some disadvantages. For example, the animals should undergo a training period, which can take from 2 d to a week, depending on how well the animals adapt to the new vehicle/food. Also, during the testing, mice may require individual housing to guarantee that each animal ingests the entire pellet, assuring delivery of the correct dose. Depending on the number of animals used in a study, this task could be time and space consuming. In addition, mice may acquire conditioned taste aversion if the drug causes adverse effects or they may refuse to eat if the vehicle used does not mask any unpleasant taste of the drug to be tested. In the end, the pros and cons of using this technique should balance animal welfare and the reliable outcomes of scientific experiments. Mice require training only once regardless of whether the test will take place in the near future or weeks later. At 10 wk after the present study, the same C57BL/6J mice were used in another experiment, with no additional training, and voluntarily ingested diet-flavored gelatin containing test compounds, indicating that interest in ingesting the gelatin pellets persisted for weeks (data not shown).

We also used gelatin pellets to test voluntary BF ingestion. In this study, the first time point for sampling started at 1 h after ingestion. For collection time points of less than 30 min, an intake time of more than 5 min may be unacceptable. However, in our study, fasted mice ate a 0.381 g gelatin pellet in less than 10 min. The speed of ingestion depends on the volume of the pellets, so the gelatin solution used to incorporate the substance must be kept to a minimum to avoid increasing the final volume of the pellet. A mouse´s stomach is comfortably full with a volume of around 0.37 ± 0.09 mL.[27] In mice, the volume of a gelatin pellet should not exceed this value to ensure a rapid voluntary intake. A smaller pellet should be easily and quickly eaten in less than 1 or 2 min.

Broccoli leaves are rich in chlorophylls and polyphenol compounds, which may confer a bitter taste.[19] In our trial, the addition of 10% sucrose to the gelatin seemed to disguise the unpleasant taste at the highest doses. At lower dose, the ingestion time was similar for unsweetened and sweetened pellets, perhaps because the BF was less concentrated, and the flavor was less intense. In this study, gavage would be more stressful because of the high volumes required and difficulties associated with administration. However, the use of gelatin pellets also has some limitations, such as the volumes or doses to be administered, the effects of an unpleasant taste of the drug, and slower ingestion that complicates measurements if shorter sampling time points are needed.

In summary, the C57BL/6J mice more readily ate gelatin, with a lower preference for lemon flavor. The use of gelatin pellets to administer drugs can be a relevant alternative technique for oral dosing of mice. However, each animal must be temporally isolated for this self-administration to ensure that the entire dose is ingested. Some mouse strains may be more suitable than others for this type of self-administration, or may need a longer period of habituation to ensure that they eat the whole gelatin pellet in a short time. Furthermore, the oral administration of a substance depends on its physical and pharmacological characteristics, and a stability study should be carried out on each substance to determine whether its incorporation into gelatin compromises its stability.

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