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Gaëlle Boudry [a,*,1], Ilario Mennella [a,1], Olivia Menard [b], Régis Janvier [a], Isabelle Nogret [a], Ashkan Madadlou [b,2], Armelle Cahu [a], Laurence Le Normand [a], Eric Bobillier-Chaumont [a], Rosalia Ferracane [c], Paola Vitaglione [c], Didier Dupont [b], David Val-Laillet [a,1]

[a] Institut Numecan, INRAE, INSERM, Univ Rennes, F-35000 Rennes, France
[b] UMR STLO INRAE, Institut Agro, F-35000 Rennes, France
[c] Department of Agricultural Sciences, University of Naples "Federico II", 80055 Portici, Italy

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ABSTRACT

Oleoylethanolamide (OEA) is a safe bioactive lipid that demonstrated strong anorexigenic properties in preclinical and clinical models. In order to evaluate the importance of OEA delivery kinetic on its anorectic properties, we developed OEA-containing dairy snacks with either a liquid or a semi-solid form. The OEA+ liquid snack, but not the semi-solid one, reduced by 14 and 18 % the amount of feed eaten by normal-weight and obese minipigs, respectively, in an eating behavior test performed 4 h after snack ingestion. In vitro digestion experiments revealed that OEA release in intestinal digesta was greatly enhanced when the snack was liquid compared to the semi-solid structure. Kinetic investigations of several plasma parameters after liquid snack ingestion points towards different potential mechanism depending upon the minipig weight status, with an effect of the OEA+ liquid snack likely on endocannabinoid and other related N-acylethanolamine metabolism in normal-weight minipigs and on ketogenesis in obese ones.

1. Introduction

The development of nutritional solutions for the prevention and/or treatment of obesity represents a direct answer that food companies could provide to support "health by food" for body weight and food intake control. In this context, oleoylethanolamide (OEA), a safe bioactive lipid, has attracted much attention from the food and pharmaceutical industries (Brown et al., 2017). Indeed, OEA demonstrated strong anorexigenic properties in mouse and rat models (Brown et al., 2017), either after intraperitoneal injection (Fu et al., 2003; Gaetani et al., 2003; Karimian Azari et al., 2014; Lan et al., 2009; Proulx et al., 2005; Rodríguez de Fonseca et al., 2001) or orally ((Oveisi, 2004). In humans, research studies highlighted correlations between endogenous plasma OEA and measures such as hunger, satiety or food palatability (Grosshans et al., 2014; Mennella et al., 2015; Monteleone et al., 2016; Rigamonti et al., 2015). Moreover, a positive effect of oral OEA supplementation twice a day for 8 or 12 weeks on the dietary intakes of obese patients was also reported (Laleh et al., 2018; Payahoo et al., 2019; Tutunchi et al., 2020).

OEA can be obtained directly from food sources, yet at very low levels (De Luca et al., 2019). Thus, the average N-acylethanolamines (NAE), which include OEA but also linoleylethanolamide (LEA) and palmitoylethanolamide (PEA), daily intake from food sources can be estimated around 0.08 mg per day for Western diet consumers and 0.25 and 0.28 mg per day for Mediterranean and vegetarian diet consumers, respectively (De Luca et al., 2019). OEA can also be endogenously formed from the omega-9 monounsaturated fatty acid, oleic acid, and an oleic acid-enriched diet has been shown to increase OEA levels in several tissues both in animal models and humans (Bowen et al., 2017). Finally, relatively pure OEA is commercially available and has been

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, N-arachidonoylethanolamine; EC, endocannabinoid; FAAH, fatty acid amide hydrolase; LEA, linoleylethanolamide; NAE, N-acylethanolamine; NEFA, non-esterified fatty acid; OEA, oleoylethanolamide; PEA, palmitoylethanolamide.

* Corresponding author at: UMR Numecan, 16 Le Clos 35590 Saint-Gilles, France.
E-mail address: Gaelle.Boudry@inrae.fr (G. Boudry).
[1] Authors contributed equally.
[2] Present address: Department of Biotechnology and Food Science, Norwegian University of Science and Technology (NTNU), Trondheim, Norway.

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incorporated into capsules for clinical trials and eventually used as dietary supplement, designed to be taken in addition to daily food consumption. Another way of consuming OEA would be to incorporate it as an ingredient in a specific tailored functional food. One question to answer first in the development of such an OEA-containing functional food is that of OEA bioavailability and timing of delivery within the organism. Indeed, in rats, the effect of OEA intraperitoneal injection is short-lasting (between 0.5 and 2 h after injection (Gaetani et al., 2003; Karimian Azari et al., 2014; Proulx et al., 2005; Rodríguez de Fonseca et al., 2001) but when delivered orally through capsules, the OEA effect on food intake was observed only after 5 h in the same species (Oveisi, 2004). Likewise, in mice, one study reported a long-lasting effect (5 h) of OEA intraperitoneal injection (Fu et al., 2003).

One easy way to modulate dietary compounds bioavailability is to change food structure, which is known to modulate gastric emptying as well as intra-luminal pH and digestive enzyme accessibility to nutrients, thus modifying the whole digestive process (Bornhorst & Paul Singh, 2014). Noteworthy, milk structure, either liquid or semi-solid, *e.g.* rennet-gel, strongly impacts digestion kinetic up to 6 to 7 h after ingestion (Barbé et al., 2013). The aim of our work was therefore to compare dairy-based OEA-containing functional foods with two distinct structures (liquid *vs*. semi-solid) in their efficacy to modulate eating behavior in a relevant animal model for nutrition research, the Yucatan minipig. We first developed an innovative methodology to encapsulate OEA, incorporated these OEA microcapsules in dairy-based liquid or semi-solid snacks, whose effects on eating behavior were tested in normal-weight and obese minipigs. Plasma analyses were further performed. to document the physiological correlates of these behavioral effects. In parallel, dynamic *in vitro* digestions were performed to study the effect of the snack structure on OEA release.

2. Materials and methods

2.1. Fabrication of OEA-loaded zein-alginate composite particles

The OEA-loaded zein-alginate particles were fabricated through the desolvation method as already described (Ge et al., 2023; Jiang et al., 2021) with some modifications. Briefly zein (Sigma-Aldrich Z3625) was dissolved (7 % w/v) in 85 % aqueous ethanol solution, followed by dissolving (2.3 % w/v) OEA ((Bertin technologies OEA-d4, OEA 82 %, LEA 2 %, PEA 0.05 %) in the solution. Subsequently, sodium alginate (Sigma-Aldrich W201502) solution (3 % w/v) in distilled water was added drop by drop into the zein-OEA aqueous ethanol solution at a volume ratio of 2.5:1. Ethanol was eliminated using a rotavapor (30 min., 100 rpm) combined with a water bath at 40 °C. The generated OEA-loaded particles were washed by distilled water and the suspension was centrifuged for 20 min. at 3000 rpm. The sedimented particles were dried at 37 °C for 12 h. The film obtained was milled in a powder (OEA+) and then used in the test food. An alginate-zein complex not including OEA (OEA-) was prepared following the same procedure and used in the control food.

2.2. Snack preparation

Four snacks (semi-solid containing OEA- or OEA+, liquid containing OEA- or OEA+) were prepared as following: 100 g of skim milk powder were dissolved in 200 g of distilled water, OEA+ or OEA- powder was also dissolved and then 180 μL of rennet (deactivated in case of the liquid food) were added and the food kept at 37 °C for 15 min. The amount of the OEA+ powder to be added was calculated for each animal in order to guarantee 25 mg of OEA per kg of body weight. Consequently, the same amount of OEA- was added in the control food. Skim milk was used to avoid any potential presence of OEA in the lipid fraction of milk.

2.3. In vitro digestion experiments

All chemicals and enzymes used for digestions were purchased from Merck, France, Sigma Aldrich (Saint Quentin Fallavier, France), unless otherwise stated.

The two snacks, dairy-based liquid and semi-solid, containing OEA were submitted to the gastrointestinal digestion system DIDGI®, INRAE, France. The computer-controlled system was set up to simulate the gastrointestinal digestion at the adult stage using parameters adapted to simulate either dairy-based liquid or semi-solid meal (Egger et al., 2019; Macierzanka et al., 2020). Before starting the digestion, 24 mL of simulated gastric fluid adjusted at pH 2 were introduced in the artificial stomach to simulate the fasted state. The gastric and intestinal emptying followed an exponential equation, as described previously (Elashoff et al., 1982). The gastric emptying half-time were set at 35 and 70 min and the β coefficient at 1.15 and 2 for liquid and semi-solid, respectively for the gastric phase (Minekus et al., 1995). The intestinal phase was longer for the semi-solid than for the liquid meal; the intestinal emptying half-times were 85 and 180 min and the β coefficient 1.4 and 1.6 for liquid and semi-solid meals respectively (Malagelada et al., 1976).

The evolution of gastric pH for the liquid ($pH = 1.68+ 3.82^{(-t/65)}$) and semi-solid ($pH = 1.68+ 4.32^{(-t/65)}$) snacks followed exponential equations where t represented the time in minute after ingestion (Malagelada et al., 1976). The pepsin solution was prepared to cover 2000 U/mL of gastric content and the amount of pepsin needed was calculated based on the activity measured in the powder. The intestinal pH conditions, pH, bile and pancreatin concentrations were set up, as previously reported by Minekus 1995. Intestinal pH was maintained at 6.7 by addition of $NaHCO_3$ 1 mol/L.

Samples were collected after intestinal emptying and kept on ice. Representative aliquots of each hour of digestion (two aliquots per period) were collected up to 4 h for the liquid meal and 6 h for the semi-solid meal. No inhibitors were added and samples were frozen at −80 °C for latter endocannabinoid (EC) and N-acetylethanolamide extraction.

2.4. Animal experiment

2.4.1. Ethical considerations

Animal experiments were conducted in accordance with the current ethical standards of the European Community (Directive 2010/63/EU), Agreement No. D3527532. The Rennes Ethics Committee in Animal Experiment and the French Ministry of Higher Education and Research have approved and authorized the entire procedure described in this paper (project number APAFIS #11984-2017103009069432_V2).

2.4.2. Housing conditions and diet

Sixteen 20-month-old male (8) and female (8) Yucatan minipigs from the INRAE UE3P animal facility (St-Gilles, France) were housed in individual stainless-steel pens (surface 110 × 80 cm, height 110 cm) equipped with metal chains to play. Bars spaced 8 cm apart allowed contact between animals in adjacent pens. Temperature was maintained at 22 °C and artificial lighting provided a 15:9 light/dark cycle. Each animal had *ad libitum* access to water. Eight (normal-weight group) minipigs received a standard diet (1.74 kcal/g, 73 kcal/kg body weight$^{0.75}$) throughout the experiment, while the eight others (obese group) received a high-fat and sucrose diet (2.58 kcal/g, 100 kcal/kg body weight$^{0.75}$) throughout the experiment (see (Gautier et al., 2020) for diet composition). They received their feed as a unique meal at 1:00 pm every day and were kept on their respective diets for 8 weeks before starting the experiments. Body weight was measured weekly throughout the protocol. One week before the onset of experiment, they were trained to eat the four snacks (on separate days) at 9:00 am within less than 15 min.

2.4.3. Surgery

A long catheter (1219.13 Leadercath; Vygon, Ecouen, France) was

inserted into the jugular vein under light anesthesia (5 μg/kg ketamide, i.m. and isoflurane inhalation (Aerane 100 mL, Baxter SAS, France) through a facial mask) two weeks before experiments.

2.4.4. Experiment 1: Impact of snacks on short-term eating behavior (Fig. 1A)

Our objective was to design a functional food that, when consumed in the morning after an overnight fast, would reduce food intake at lunch. Thus, the day of the experiment, overnight fasted minipigs were offered one of the four snacks (semi-solid OEA- or OEA+, liquid OEA- or OEA+) at 9:00am, which was consumed in less than 15 min. The amount of snack offered to the animals was calculated to deliver 25 mg of OEA per kg body weight and 440 kcal. After 4 h during which they did not receive any other feed, minipigs were exposed to their usual diet *ad libitum*. Eating tests were performed for 1 h using in-house pig-dedicated meal pattern recording troughs as already described (Ochoa et al., 2014). Minipigs received each of the four snacks in a.

2.4.5. Experiment 2: Impact of liquid snacks on endocannabinoid and anorexigenic and orexigenic factor plasma profiles (Fig. 1B)

We next evaluated different plasma profiles in the 4 h that followed the liquid snacks consumption. We focused on the liquid snacks only since the OEA+ liquid snack exhibited promising anorexigenic properties. The day of the experiment, minipigs were offered one of the two liquid snacks (OEA- or OEA+) at 9:00am, which was consumed in less than 15 min. Blood (10 mL) was drawn from the intra-jugular catheter either 20 min before or 30, 60, 120, 180 and 240 min after the snack consumption. The animals received the two snacks in a random order with a minimum of 2 days between trials. Blood was immediately placed in tubes containing either EDTA (final concentration 8 mM) for later EC, glucose, non-esterified fatty acid (NEFA), β-hydroxybutyrate and insulin analysis or EDTA (final concentration 8 mM) and anti-DPPIV (Millipore, USA,1:100 v/v) for later GLP-1 analysis, or anti-protease (aprotinin-containing tubes (BD Life Science, France)) for later ghrelin analysis. Blood was immediately centrifuged (2500g, 10 min., 4 °C) and plasma aliquoted and stored at −80 °C until analysis. For ghrelin analysis, HCl 1 N (1:20 v/v) and phenylmethanesulfonyl fluoride (1:100 v/v) were added to plasma before storage at −80 °C.

2.5. Extraction and analysis of endocannabinoids and n-acetylethanolamides

ECs and NAEs were extracted from EDTA plasma samples and from *in vitro* digestion samples. A solid-phase extraction according to the method described by Marczylo and colleagues (Marczylo et al., 2009) was carried out using Oasis HLB cartridges (1 cc/30 mg, Waters). After extraction, samples were dried under nitrogen flow and kept at −80 °C until analysis. Acetonitrile/water (50:50) 100 μL were used to reconstitute samples just before LC–MS/MS analysis performed following the method previously described (Mennella et al., 2015).

2.6. Plasma analysis

Plasma glucose, NEFA and β-hydroxybutyrate concentrations were assessed using an automated spectrophotometric method (Konelab 20i, Thermo Fisher Scientific, Illkirch, France) using specific kits (Thermo Fisher Scientific for glucose and β-hydroxybutyrate, Wako for NEFA). Plasma insulin concentrations were determined using a commercial immunoassay kit (ST AIA-PACK IRI) and the AIA-1800 device (Automated Immunoassay Analyzer; TOSOH Bioscience, Tokyo, Japan). Plasma GLP-1 concentrations were measured using a porcine-dedicated GLP-1 active ELISA kit (Millipore). Finally, plasma active (octanoyled) ghrelin concentrations were determined using dedicated radio-immunoassay kit (Phoenix Pharmaceuticals).

2.7. Statistical analysis

Data are presented as means ± standard error of the mean. Normal-weight and obese minipig data were analyzed separately. Sex effect and its interaction with other tested parameters was first tested. In lean minipigs, sex was significant for plasma LEA (M > F, P = 0.02), AEA (M > F, P = 0.001), insulin (F > M, P = 0.003) and glucose (F > M, P = 0.04). In obese minipigs, sex was significant for plasma OEA (F > M, P = 0.02), 2-AG (F > M, P = 0.001) and AEA (F > M, P = 0.02). However, for all these parameters, there was not interaction with presence of OEA in the snack. Thus, male and female data were pooled for further analysis. Meal pattern parameters were analyzed using a 2-way analysis of variance testing the snack structure (semi-solid or liquid) and OEA presence (OEA+ or OEA-) and the interaction between these two factors. Plasma parameters were analyzed using a 2-way analysis of variance testing OEA presence (OEA+ or OEA-), time and the interaction between these two factors. Post-hoc analyses were performed using Sidak test when appropriate.

3. Results

3.1. Liquid OEA-enriched dairy snack reduced short-term feed intake

To investigate the impact of OEA-enriched dairy snack on feed intake, we designed OEA-enriched (OEA+) or control (OEA-) snacks with different structures, either in a liquid form (*i.e.* milk) or a semi-solid form (*i.e.* curd obtained after rennet coagulation). Overnight fasted normal-weight and obese minipigs were offered the four snacks in a random order and their eating behavior investigated 4 h after snack consumption (Fig. 1**A**). Irrespective of the type of snack and weight status, all the minipigs displayed a hyperphagic behavior with immediate eating onset and a very long (average duration 2920 s [min: 1168 s - max: 3599 s], *i.e.* nearly 49 min) and large (average amount 1712 g [min: 560 g - max: 3034 g]) first meal.

The amount of feed was significantly reduced after minipigs had consumed the OEA+ snack in the liquid form compared to the liquid OEA- snack, irrespective of the minipig weight status (OEA+ *vs.* OEA- liquid: normal weight minipigs −14 %, P = 0.016, obese minipigs −18 %, P = 0.018, Fig. 2**A**). In addition, in obese minipigs, the amount of feed ingested was greater after OEA+ semi-solid snack compared to OEA- semi-solid snack ingestion (+27 %, P = 0.018), although this was primarily due to a low feed intake after the OEA- semi-solid diet consumption compared to the other control conditions. Duration of the first meal was not different after the OEA- and OEA+ snacks, irrespective of the minipig weight status or the snack structure (Fig. 2**B**).

3.2. Endocannabinoid and related NAEs release from the snack was greater for the liquid than the semi-solid snack in in vitro digestion experiments

To understand why OEA+ snacks with different structures induced different eating behaviors, we evaluated EC release from the liquid and semi-solid OEA+ snacks in *in vitro* digestion experiments. Luminal samples were taken every hour for 6 h (semi-solid snack) or until complete emptying of the gastric compartment, *i.e.* after 4 h (liquid snack). Endocannabinoid and related NAEs (OEA but also LEA, PEA and AEA since minor percentages of these compounds were present in the initial OEA powder) concentrations were analyzed in these samples and cumulative EC concentrations calculated for each snack. The concentrations of OEA, LEA and PEA increased with digestion time for both types of snacks but concentrations were largely increased (x 357, 152 and 38 for OEA, LEA and PEA, respectively after 4 h of digestion) for the liquid compared to the semi-solid snack, starting to plateau after 3 h of digestion (Fig. 3 **A-C**). The concentrations of AEA were below detectable levels. Thus, the difference in eating behavior after liquid or semi-solid OEA+ snacks ingestion could account for the difference in EC release

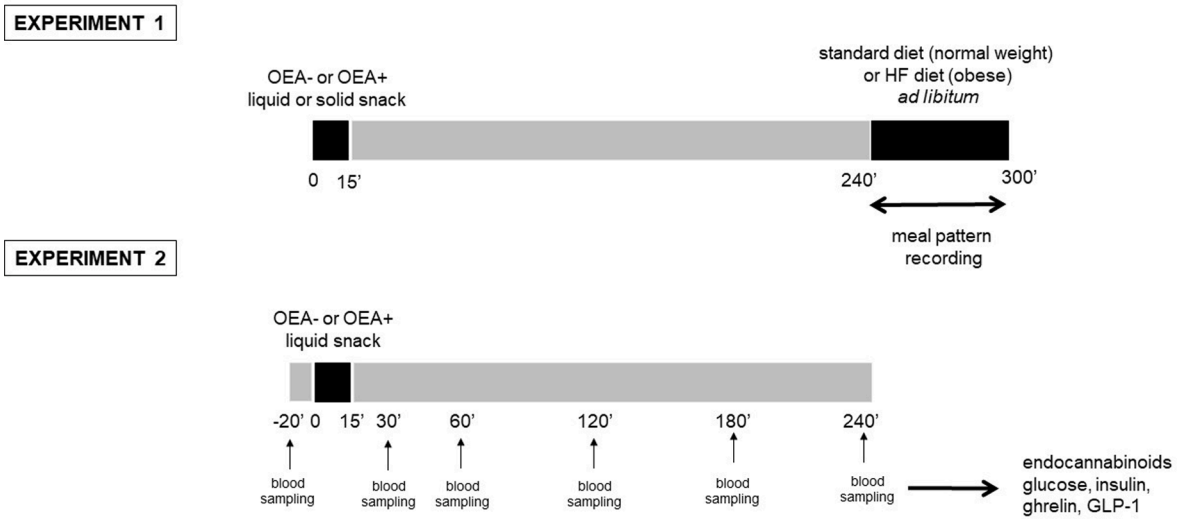

Fig. 1. Overview of the animal experiment design.

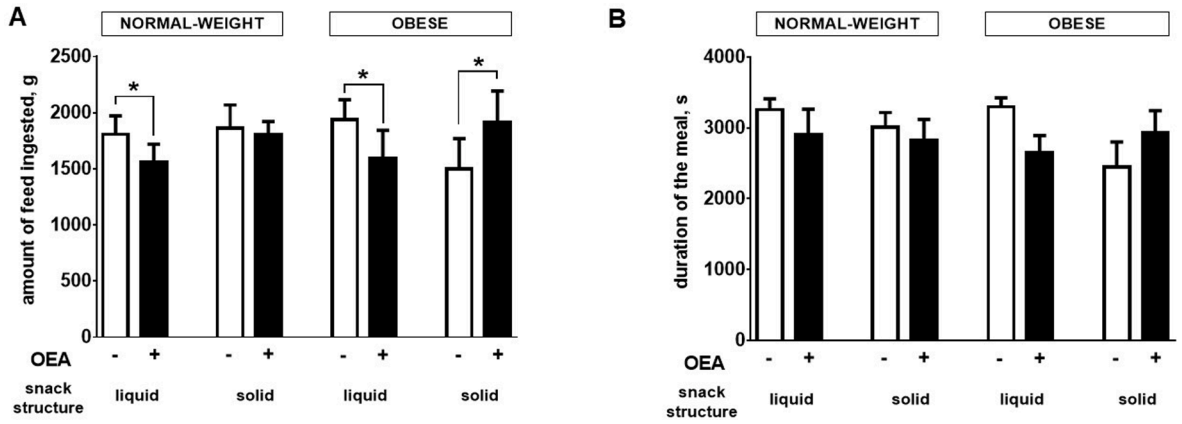

Fig. 2. Impact of OEA-enriched snack on short-term eating behavior in normal-weight and obese minipigs. Overnight fasted normal-weight and obese minipigs were fed the OEA enriched (OEA+) or control (OEA-) snack in liquid or solid form. After 4 h, they were allowed access to feed. The amount of feed ingested (A) and duration (B) of the first meal were recorded. All pigs received the four snacks (liquid OEA-, liquid OEA+, solid OEA-, solid OEA+) in a random order with a minimum of 2 days between tests. Values are means ± SEM, * $P < 0.05$, n = 8 normal-weight and 8 obese minipigs.

during the digestive process *in viv*.

3.3. Presence of OEA in the dairy liquid snack reduces 2-AG plasma level in normal-weight but not obese animals

To further understand the effect of the liquid OEA+ snack, we next investigated plasma EC profiles during the 4 h following the liquid OEA- or OEA+ snack ingestion. We focused on the liquid snacks only since only this snack structure exhibited anorexigenic properties (Fig. 1**B**). There was no significant difference in fasted EC plasma levels (2-arachidonoylglycerol (2-AG), LEA, PEA, N-arachidonoylethanolamine (AEA) and OEA) between normal-weight and obese minipigs, except for a tendency for greater AEA plasma level in obese animals (Table 1). After liquid snack ingestion, the levels of OEA, 2-AG, LEA and PEA, but not AEA, significantly dropped in normal-weight minipigs (Fig. 4**A, C, E, G, I**). On the contrary, no variation of plasma levels was noticed for these ECs in obese minipigs (Fig. 4**B, D, H, J**), except AEA levels, which dropped after the liquid snack ingestion (Fig. 4**F**). There was no difference in EC levels after OEA- or OEA+ liquid snack ingestion, irrespective of the animal weight status, except for 2-AG plasma level, which was lower 4 h after the liquid OEA+ snack compared to the OEA- one in normal-weight minipigs (-52 %, $P = 0.034$, Fig. 4**C**).

3.4. Liquid OEA-enriched snack altered plasma anorexigenic factors differently depending on the minipig weight status

We next investigated whether liquid OEA+ snack modified various known anorexigenic and orexigenic factors such as GLP-1 or plasma glucose, insulin (anorexigenic factors) and active ghrelin (orexigenic). Obese minipigs exhibited higher glycemia, insulinemia and plasma β-hydroxybutyrate level in the fasting state than normal-weight minipigs but no difference for plasma active ghrelin, GLP-1 or NEFA was observed (Table 1).

In normal-weight minipigs, plasma GLP-1 levels did not vary after liquid snack consumption, irrespective of the presence of OEA (Fig. 5**A**). On the contrary, in obese minipigs, consumption of the liquid OEA+ snack resulted in a lower plasma GLP-1 level after 4 h compared to basal value ($P = 0.049$, Fig. 5**B**). Consumption of the liquid OEA- snack did not affect plasma GLP-1 levels in obese animals. No significant difference in GLP-1 levels between the two snacks was observed at any timepoint.

Insulinemia sharply increased 30 min after the liquid OEA- snack consumption in normal-weight minipigs ($P = 0.002$, Fig. 5**C**) but not after the liquid OEA+ snack consumption. Insulinemia had returned to normal value at 60 min. In obese animals, liquid snack consumption induced a greater and longer increase in plasma insulin than in normal-weight minipigs, with a significant increase of insulinemia compared to

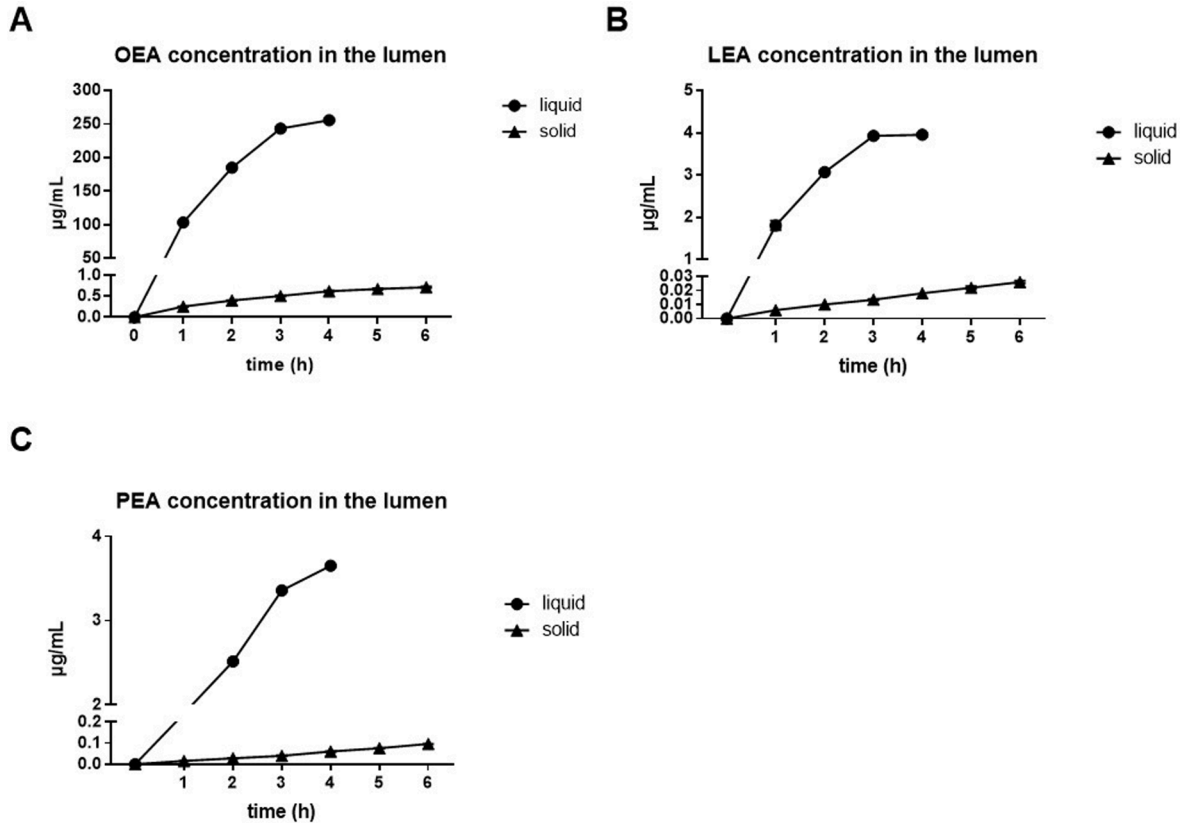

Fig. 3. Concentrations of endocannabinoids and related N-acylethanolamines in the intestinal luminal compartment during snack *in vitro* digestion tests. OEA+ -liquid and solid snacks were submitted to *in vitro* gastro-duodenal digestions to measure endocannabinoid (A: oleoylethanolamide (OEA), B: linoleylethanolamide (LEA), C: palmitoylethanolamide (PEA)) concentrations in the intestinal luminal compartment.

Table 1
Fasted plasma endocannabinoid and related NAEs and anorexigenic and orexigenic factors in normal-weight and obese minipigs.

	Normal-weight	Obese	P-value
2-AG, ng/mL	5.4 ± 1.7	2.6 ± 0.8	0.20
LEA, ng/mL	2.9 ± 1.3	0.9 ± 0.3	0.23
PEA, ng/mL	12.7 ± 2.1	9.0 ± 1.2	0.19
AEA, ng/mL	0.16 ± 0.02	0.32 ± 0.08	0.06
OEA, ng/mL	26.3 ± 7.8	16.0 ± 9.4	0.29
Glucose, mM	4.4 ± 0.08	4.9 ± 0.04	< 0.0001
Insulin, µU/mL	7.6 ± 1.4	14.8 ± 1.6	0.005
GLP-1, pM	18.1 ± 6.3	32.5 ± 12.7	0.31
Active ghrelin, pg/mL	78.7 ± 10.0	71.6 ± 6.0	0.57
NEFA, mM	0.11 ± 0.02	0.11 ± 0.03	0.90
β-hydroxybutyrate, mM	0.03 ± 0.008	0.08 ± 0.01	0.01

Data are means ± SEM. N = 8 normal-weight and 8 obese minipigs. 2-AG: 2-arachidonoylglycerol, LEA: linoleylethanolamide, PEA: palmitoylethanolamide, AEA: arachidonoylethanolamine, OEA: oleoylethanolamide, GLP-1: glucagon-like peptide, NEFA: non-esterified fatty acids.

basal level 30 and 60 min after liquid OEA+ snack consumption (P = 0.01 and 0.004, respectively, Fig. 5**D**).

In parallel to insulinemia, plasma glucose level dropped 30 min after both liquid OEA- and OEA+ snack consumption, (P = 0.003 and 0.005, respectively, Fig. 5**E**) in normal-weight minipigs. It then increased to levels higher than normoglycemia at 120 and 180 min only after the liquid OEA- snack ingestion (P = 0.04 and 0.01, respectively) while it had returned to a normal level as soon as 60 min after liquid OEA+ snack ingestion (Fig. 5**E**). Contrary to what happened in normal-weight minipigs, no variations in glycemia after snack consumption was observed in obese minipigs, irrespective of the presence or OEA or not (Fig. 5**F**).

In normal-weight minipigs, plasma active ghrelin level decreased after liquid snack consumption to reach a significantly different level than the basal one after 4 h (P = 0.001 for both snacks, Fig. 5**G**), with no difference between OEA- and OEA+ liquid snacks. In obese minipigs, no significant variation of the plasma ghrelin level was observed after liquid snack ingestion, irrespective of the presence of OEA (Fig. 5**H**).

Plasma NEFA levels dropped after both liquid snack ingestion in normal-weight minipigs, irrespective of the presence of OEA (Fig. 5**I**) but did not vary with time in obese minipigs, irrespective of OEA presence in the liquid snack (Fig. 5**J**). Finally, plasma β-hydroxybutyrate levels did not vary with time in normal weight minipigs, irrespective of the snack (Fig. 5**K**). However, it increased in obese minipigs after ingestion of the OEA- liquid snack, reaching a plateau at 120 min while it did not change after OEA+ liquid snack ingestion, resulting in a significant difference in plasma β-hydroxybutyrate levels between the two snacks at 120 and 180 min (Fig. 5**L**).

4. Discussion

Our objective was to design a dairy-based OEA-containing functional food that, when consumed in the morning after an overnight fast, would reduce food intake at lunch. Our liquid OEA+ snack exhibited promising anorexigenic properties since it reduced the amount of feed consumed by both normal-weight and obese minipigs while the solid snack did not display such effects, a difference that could be accounted for by the higher EC release observed during *in vitro* digestion experiments with the liquid OEA+ snack compared to solid OEA+ snack. Yet, the mechanisms of effect of the OEA- liquid snack on feed intake seems to be dependent upon the weight status since in normal-weight mini-pigs, difference in 2-AG level could account for the anorexigenic effect of the OEA+ liquid snack, while in obese minipigs, an effect of the OEA+ liquid snack on ketogenesis could be at play.

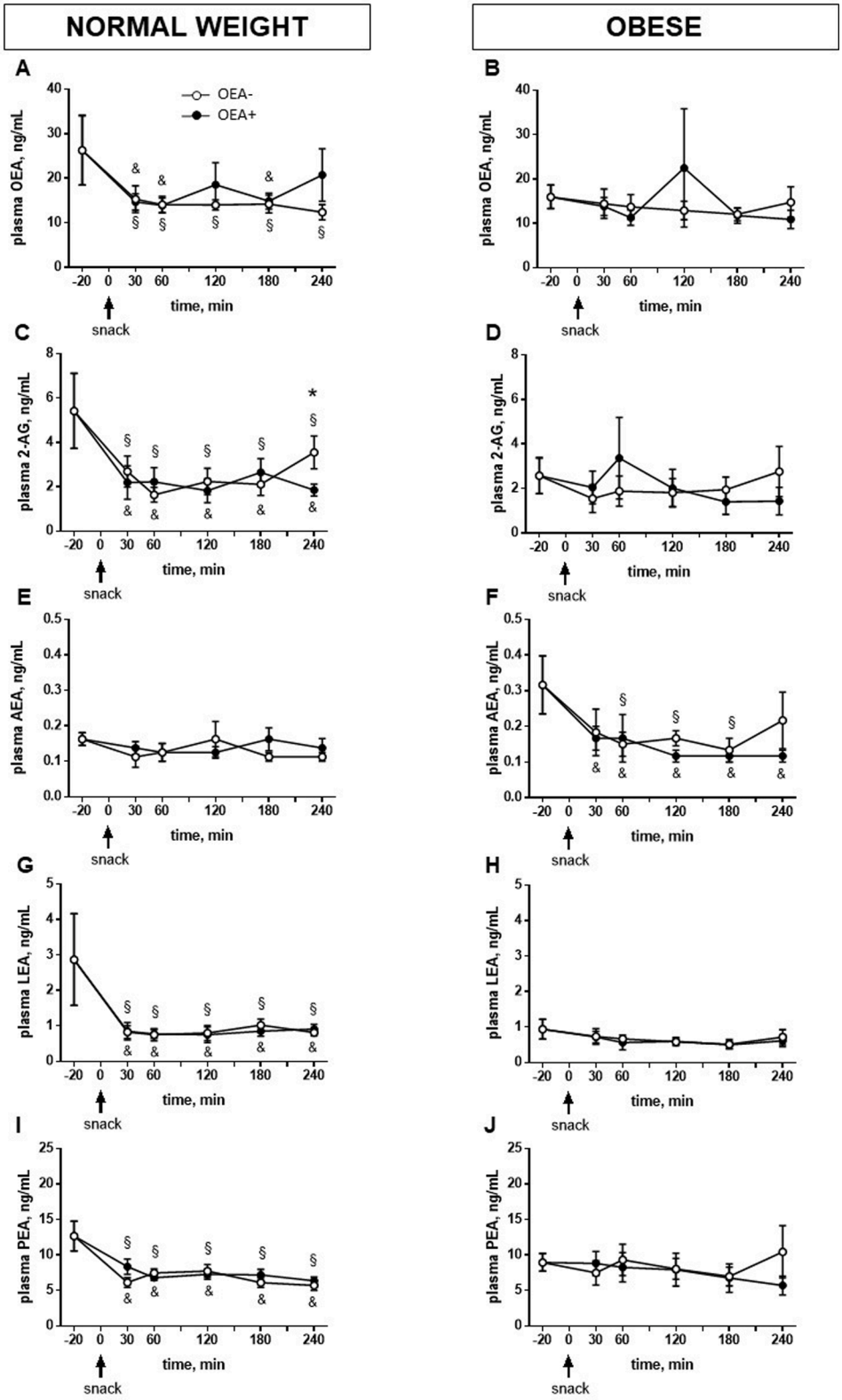

Fig. 4. Plasma endocannabinoid and related acylethanolamides profiles following OEA-enriched or control liquid snack ingestion in normal-weight and obese minipigs. Plasma ECs and related NAEs (A-B: oleoylethanolamide (OEA), C-D: 2-arachidonoylglycerol (2-AG), E-F: arachidonoylethanolamine (AEA), G-H: linoleylethanolamide (LEA), I-J: palmitoylethanolamide (PEA)) levels were determined in normal-weight and obese minipigs after ingestion of the OEA- (open circles) or OEA+ (solid circles) liquid snacks. Values are means ± SEM, * $P < 0.05$ OEA- *vs.* OEA+, § $P < 0.05$ T0 *vs.* other timepoints for OEA- snack, & $P < 0.05$ T0 *vs.* other timepoints for the OEA+ snack. N = 8 normal-weight and 8 obese minipigs.

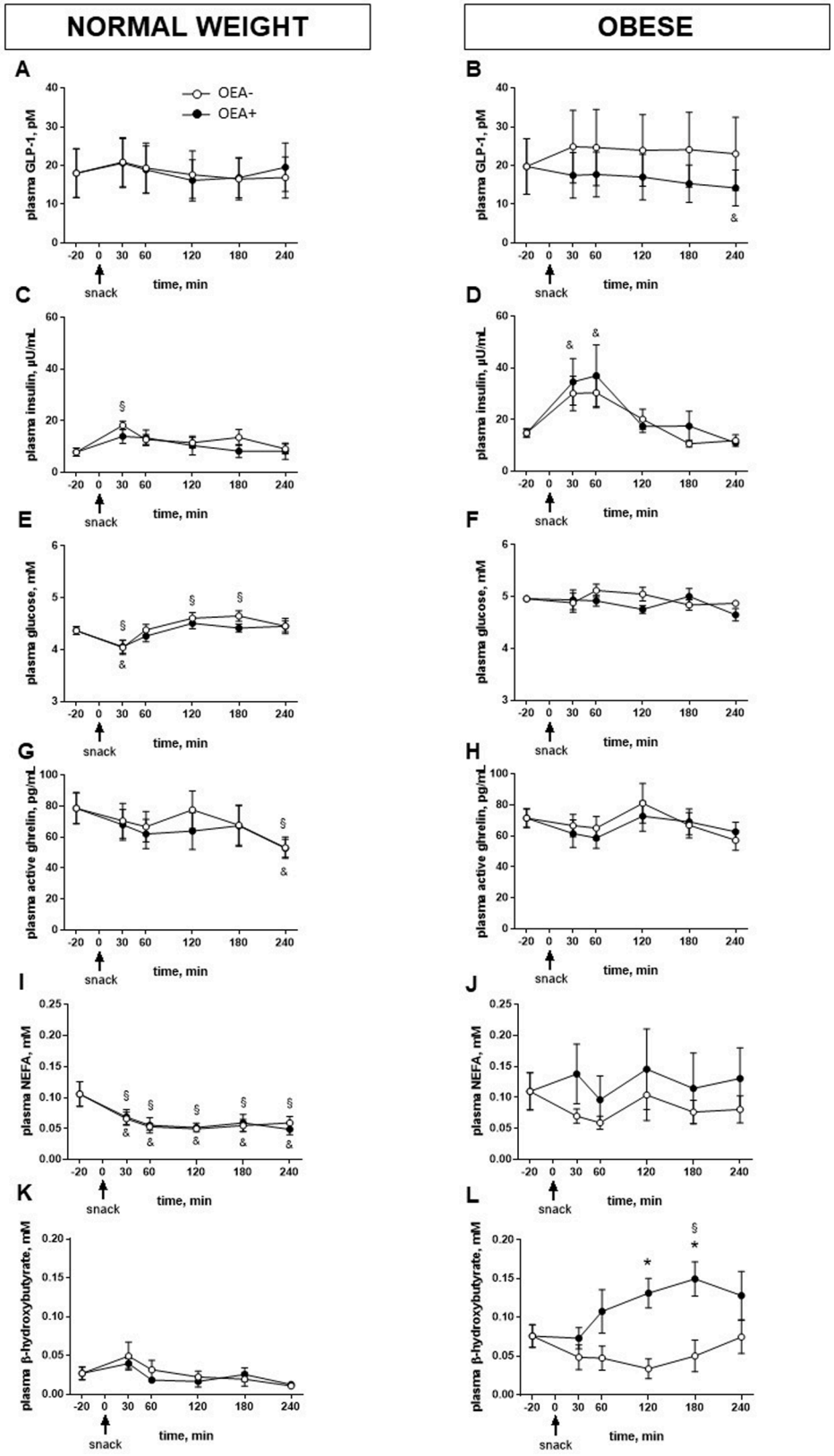

Fig. 5. Plasma anorexigen and orexigen factor profiles following OEA-enriched or control liquid snack ingestion in normal-weight and obese minipigs. Plasma GLP-1 (A-B), insuline (C-D), glucose (E-F), ghrelin (G-H), non-esterified fatty acid (NEFA) (I-J) and β-hydroxybutyrate (K-L) levels were determined in normal-weight and obese minipigs after ingestion of the OEA- (open circles) or OEA+ (solid circles) liquid snacks. Values are means ± SEM, § $P < 0.05$ T0 *vs.* other timepoints for the OEA- snack, & $P < 0.05$ T0 *vs.* other timepoints for the OEA+ snack. N = 8 normal-weight and 8 obese minipigs.

To evaluate the effect of OEA-containing snacks, we chose to perform our study in adult minipigs since this animal species is close to humans in terms of digestive physiology, eating behavior and brain functions (Roura et al., 2016). This species exhibits a functional EC system (synthesis and degrading enzymes, receptors) both at the gut (Toschi et al., 2020, 2021) and central (Goparaju et al., 1999; Pirone et al., 2020) levels. Yet OEA effect had never been tested *in vivo* in pigs before. We observed a progressive decrease of plasma levels of 2-AG, OEA and PEA after snack ingestion in normal-weight minipigs as described post-prandially in normal-weight healthy humans (Monteleone et al., 2016). Altogether, this makes the pig a suitable model to evaluate OEA effects upon eating behavior. Moreover, we evaluated the effect of OEA+ snacks in both normal-weight and obese minipigs. Several reports indicated that OEA metabolic and anorixegenic effects differed between lean and obese status in rodent models (Bowen et al., 2017; Brown et al., 2017), which justifies testing the OEA+ snacks in both weight status. Although we did not thoroughly characterize the possible differences in the EC system in these animals, obese minipigs displayed features of altered glucose and lipid metabolism (higher fasted glycemia, insulinemia and NEFA plasma levels) but also a tendency for increased AEA plasma levels as already described in obese patients (Engeli et al., 2005). Moreover, no decrease of EC plasma levels, except for AEA, was observed in our obese minipigs after the snack ingestion as opposed to normal-weight minipigs and humans ((Monteleone et al., 2016), suggesting altered EC system in these obese minipigs.

OEA has been shown to impact eating behavior by acting both on the homeostatic and hedonic regulation of food intake as it interferes with oxytocin and histamide signaling at the hypothalamic level, dopamide and cannabinoid receptor 1 signaling in central reward centers but also GLP-1 and ghrelin pathways at the gut-brain level (Brown et al., 2017). We tested the effect of OEA+ solid and liquid snacks in a 1-hour *ad libitum* eating test in almost fasted animals (except the snack eaten 4 h before). Noteworthy, we used the pigs' usual diets (*e.g.* standard diet for the normal-weight minipigs and high-fat and high-sugar diet for obese animals) to avoid neophobia, which has already been documented in minipigs with this type of diets (Coquery et al., 2019). This experimental set-up was first intended to evaluate the homeostatic eating parameters such as latency to eat (time between feed presentation and eating onset) that has been shown to be reduced by prior OEA intraperitoneal injection in food-deprived rats (Gaetani et al., 2003; Karimian Azari et al., 2014), meal size (objectivizing satiation) that was also reduced in OEA-treated food-deprived rats (Gaetani et al., 2003; Karimian Azari et al., 2014), inter-meal interval (objectivizing satiety) and speed of ingestion (amount of feed eaten by unit of time). However, since our minipigs were almost fasted, this eating behavior test was performed on highly feed motivated animals, which might have hidden some of the effects of OEA on homeostatic eating parameters. Nonetheless, we observed a significant effect of the OEA+ liquid snack on meal size in both normal-weight and obese minipigs, suggesting an effect on satiation as already described in rodents (Gaetani et al., 2003; Karimian Azari et al., 2014). Latency to eat was not impacted by OEA+ snacks, which might be accounted for by the high eating motivation of our pigs. Lastly, our eating behavior test was not designed to properly evaluate food motivation. Indeed, food motivation is usually tested using a progressive ratio operant task that evaluates the rewarding impact of food by measuring the animal willingness to work (*e.g.* pushing a button repeatedly) to obtain food rewards (Ferguson et al., 2009). Thus, further work is needed to evaluate precisely the impact of OEA+ snacks on motivation for feed using such dedicated tests.

The release of EC from the liquid and semi-solid snacks during dynamic *in vitro* digestion experiments was drastically different. While the digestion of the semi-solid snack ended up with a limited release of OEA, LEA and PEA, the concentrations of these three molecules released after gastrointestinal digestion of the liquid snack were considerably higher. It must be noted that the presence of LEA and PEA in the *in vitro* digesta likely results from the fact that the OEA we used was not pure and contained very small amounts of these NAEs. At that stage, only hypotheses can be raised to explain the observed differences. A possible OEA degrading effect of the chymosin present in rennet during the semi-solid snack production is unlikely. Indeed, the measured concentration of OEA in the semi-solid snack before digestion (382.7 ± 3.6 μg/mL) was very close to that in the liquid snack (427.3 ± 2.8 μg/mL) demonstrating that OEA has not been broken down by chymosin during semi-solid snack preparation. A low efficiency of the extraction protocol used for the quantification of EC in semi-solid snack digestion samples due to the structure of the food matrix itself can hardly be considered since it would have affected the quantification of OEA in the undigested semi-solid snack as well. One can also hypothesize that when arriving in the small intestine, OEA delivered via the semi-solid snack is more prone to a fast hydrolysis by enzymes than with the liquid snack. Fatty acid amide hydrolase 1 and 2 (FAAH-1 and 2) and N-acylethanolamine-hydrolyzing acid amidase are abundant in the small intestine *in vivo* (Bowen et al., 2017). However, the proteome of pancreatin that was used in the present study to simulate the small intestinal digestion phase has been recently published (Wang et al., 2022) and none of these enzymes were identified. Another possibility is that OEA in liquid snack that is rapidly transferred from the stomach to the small intestine is subject to a limited oxidation by the oxygen present in the stomach cavity. In contrast, OEA in the semi-solid snack stayed longer in the stomach due to slower gastric emptying rate resulting in increased oxidation. Thus, further investigations are needed to understand the difference of EC release with snack structure.

We next sought to unravel the mechanism by which the liquid OEA+ snack reduced food intake in our minipigs. We first established plasma EC profiles during the 4 h following the liquid snack ingestion and preceding the meal tests. Despite the presence of 20 mg/kg of BW of OEA in the OEA+ snack, no increase in plasma OEA levels was noticed, irrespective of the minipig ponderal status. On the contrary, plasma OEA levels decreased after snack ingestion in normal-weight minipigs, as well as those of PEA, 2-AG and LEA, as already described post-prandially in humans (Monteleone et al., 2016). Absence of increase in plasma OEA levels following OEA ingestion or stimulation of OEA endogenous synthesis has already been described, leading some authors to consider that exogenous OEA is poorly absorbed and that plasma OEA is a spillover from OEA-producing organs (Brown et al., 2017). Noteworthy, ECs, including OEA, are degraded by FAAH, an intra-cellular enzyme that is highly present in the intestinal epithelium (Katayama et al., 1997). Thus, the absence of increase of OEA plasma levels might account for by a high rate of degradation in the intestine. We also established the plasma levels of OEA relative compound (2-AG, LEA PEA and AEA) after liquid snack ingestion. Interestingly, plasma 2-AG was lower 4 h after liquid OEA+ snack ingestion than after OEA- ingestion in normal-weight minipigs. 2-AG has opposite effect compared to OEA as it increases food intake due to its affinity for CB1 receptors (Lau et al., 2017). Thus, the lower plasma 2-AG level in normal-weight minipigs 4 h after liquid OEA+ snack ingestion compared to the liquid OEA- snack (*i.e.* at the moment of the meal test) might have a role in the reduced food intake observed with this OEA+ snack in these animals, although this warrants further investigations.

We also speculated that other mechanisms might be involved than a direct effect of circulating ECs on the brain. Endogenous OEA has been shown to bind to GLP-1 receptors, potentiating GLP-1 effect (Cheng et al., 2015). We therefore measured plasma GLP-1 levels after liquid OEA+ and OEA- snack ingestion, which did not vary in normal-weight minipigs and even decreased 4 h after the OEA+ snack ingestion in obese minipigs, probably because of the low energy and/or nutrient composition of this snack. To evaluate the potentiating effect of OEA upon GLP-1 receptor, we investigated the GLP-1 incretin effect which is mediated by GLP-1 receptor at the pancreatic level (Reed et al., 2020), hypothesizing that the OEA+ snack would increase plasma insulin levels and/or reduce plasma glucose levels more efficiently than the OEA- snack. However, variations in glycemia and insulinemia after snack

ingestion were similar between OEA+ and OEA- snacks, ruling out the hypothesis of OEA-mediated enhancing of GLP-1 action at the central level. Noteworthy, the variations in glycemia and insulinemia were very small, probably due to the low glycemic index of milk that composed the snack and in agreement with a recent human study showing the post-prandial hormonal response following milk consumption in healthy subjects (Tagliamonte et al., 2023). We also investigated the ghrelin pathway since OEA had been shown to reduce ghrelin levels in fasted rats (Serrano et al., 2011) and that OEA and ghrelin levels were correlated in a palatable food anticipatory and consummatory test in obese humans (Rigamonti et al., 2015). Yet, no difference in plasma ghrelin levels was observed after liquid OEA+ or OEA- snack ingestion, also excluding this hypothesis.

Lastly, we explored the potential effect of OEA on lipid metabolism. Indeed, OEA is a high-affinity endogenous ligand of the nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR-α) and as such stimulates lipolysis and fatty acid β-oxidation, both known to act as anorexigenic signals (Langhans et al., 1985). Circulating NEFA and ketone bodies, enhanced during fasting to provide energy to energy-demanding organs, usually decrease after eating, mainly due to the inhibitory action of insulin on lipolysis and the concomitant stimulation of lipogenesis, which, in turn, inhibits fatty acid β-oxidation. This decrease in plasma NEFA after snack ingestion was observed in normal-weight animals but not obese ones, showing one again alteration of metabolism in obese minipigs. Yet, NEFA plasma level did not differ between OEA- and OEA+ liquid snacks in both normal weight and obese minipigs, ruling out an effect of OEA on lipolysis in our case. Interestingly, in obese animals, β-hydroxybutyrate, a marker of ketogenesis, increased after the OEA- liquid snack consumption but not the OEA+ one. In metabolically healthy animals, ketone bodies contribute to inhibition of eating (Langhans et al., 1985). Thus, this lower plasma level of β-hydroxybutyrate after OEA+ liquid snack ingestion in obese minipigs and the reduced food intake in the eating behavior test are counterintuitive. Yet, the impact of ketone bodies on appetite regulation in obese individuals is poorly described. Noteworthy, fasting plasma β-hydroxybutyrate level was higher in obese than normal-weight minipigs, indicating altered ketogenesis in obese animals, as already observed in mice chronically fed a high-fat diet (Sunny et al., 2010). Increased ketogenesis after the OEA- liquid snack ingestion also probably reflects aberrant metabolic responses in obese animals, that seems to be counteracting by the presence of OEA in the snack. Further investigations are therefore needed to evaluate if the OEA+ liquid snack effect on food intake in obese animals is mediated by an effect on ketogenesis.

This princeps preclinical trial in Yucatan minipigs demonstrated the ability of an OEA supplemented functional dairy liquid snack to decrease food intake during a meal test performed 4 h later in fasted and highly-motivated normal-weight and obese individuals. A semi-solid snack (*i.e.* curd) did not produce such effects on feed intake, nor the significant liberation of OEA, LEA and PEA observed with the liquid snack in *in vitro* digestors. We must acknowledge that the OEA powder we used in our study was not pure OEA and that, even if they were present in very small proportion (<2%), other NAEs could also have played a role in the observed anorexigenic effect of the liquid snack. Moreover, our eating behavior tests were performed in highly feed motivated animals that prevented us from properly evaluate satiation and satiety effects of our snacks. We also did not evaluate the long-lasting effects of the snacks on eating behavior. Finally, our mechanistic investigations point towards an effect of the OEA+ liquid snack on plasma 2-AG level in normal-weight animals and on ketogenesis in obese animals to explain this anorexigenic effect. Yet, further experiments are needed to firmly conclude on the effect of our functional snack, which might modulate both homeostatic and hedonic brain circuits involved in the control of food intake and motivation.

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Author contribution

GB, IM, DVL: designed and directed the project, carried out experiments, drafted the manuscript; PV, DD: discussed resulted, reviewed the manuscript; OM, RJ, IN, AM, AC, LLN: carried out the experiments and performed the analysis, reviewed the manuscript, EBC: designed and manufactured equipment (eating microstructure troughs), reviewed the manuscript.

Ethic statement

Animal experiments were conducted in accordance with the current ethical standards of the European Community (Directive 2010/63/EU), Agreement No. D3527532. The Rennes Ethics Committee in Animal Experiment and the French Ministry of Higher Education and Research have approved and authorized the entire procedure described in this paper (project number APAFIS #11984-2017103009069432_V2).

CRediT authorship contribution statement

Gaëlle Boudry: Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Ilario Mennella:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Olivia Menard:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Régis Janvier:** Writing – review & editing, Investigation. **Isabelle Nogret:** Writing – review & editing, Investigation. **Ashkan Madadlou:** . **Armelle Cahu:** Writing – review & editing, Investigation. **Laurence Le Normand:** Writing – review & editing, Investigation. **Eric Bobillier-Chaumont:** Writing – review & editing, Methodology. **Rosalia Ferracane:** Writing – review & editing, Methodology. **Paola Vitaglione:** . **Didier Dupont:** . **David Val-Laillet:** .

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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