The umami taste in pigs:

L-amino acid preferences and
in vitro recognition by the receptor
dimer pT1r1/pT1r3 expressed
in porcine taste and non-taste tissues

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Glossary

AA	Amino acids
ADFI	Average daily feed intake
ADG	Average daily gain
AgRP	Agouti related protein
AMPK	Adenosine monophosphate-activated protein kinase
ANF	Anti-nutritional factors
AP	Area postrema
ARC	Arcuate nucleus
AS	Attempts to escape
ASICs	Acid-sensing ion channels
B/G	Blue green ratio
Bla	β-lactamase
BW0	Initial body weight
BW20	Final body weight
CaR	Calcium receptor
CART	Cocaine- and amphetamine-related transcript
CCK	Cholecystokinin
CHO	Chinese hamster ovary
CNS	Central nervous system
CP	Crude protein
CRT	Consumption rate of test solution
CT	Chorda Tympani nerve
Ct	Cycle thresholds
D-AA	D-amino acids
D,L-Met	D,L-Methionine
DAG	Dyacilglicerol
DOCH	Double-choice
EAA	Essential amino acids
FG	Feed to gain ratio

FRET	Fluorescence resonance energy transfer
GABA	Gamma amino butyric acid
GEE	Generalized estimating equations
GI	Gastrointestinal
GLP-1	Glucagon-like peptide 1
GLUT2	Glucose transporter type 2
Gly	Glycine
GMP	Guanosine-5'-monophosphate
GP	Glossopharyngeal nerve
GPCRs	Guanine-coupled-nucleotide-binding protein coupled receptors
GPRC6A	G-protein coupled-receptor family C group 6 member A
GSP	Greater superficial petrosal nerve
G_	Gustducin
H	Sour
HCN	Hyperpolarization-activated cyclic-nucleotide-gated channels
HCP	High crude protein diet
HEK	Human embryonic kidney
HTS	High throughput screening assays
IMP	5'-inosine monophosphate
IP3	Inositol triphosphate
K2P	Potassium channels
L-AA	L- amino acids
L-Ala	L-Alanine
L-Arg	L-Arginine
L-Asn	L-Asparragine
L-Asp	L-Aspartic acid
L-Cis	L-Cistine
LCP	Low crude protein diet
L-Cys	L-Cysteine
L-Gln	L-Glutamine
L-Glu	L-Glutamic acid
L-His	L-Histidine
L-Iso	L-Isoleucine
L-Leu	L-Leucine
L-Lys	L-Lysine
L-Phe	L-Phenilalanine
L-Pro	L-Proline
L-Ser	L-Serine

L-Thr	L-Threonine
L-Trp	L-Tryptophan
L-Tyr	L-Tyrosine
L-Val	L-Valine
M	Mineral
ME	Median eminence
mGlu	Metabotropic glutamate receptor
mGluR1	Truncated type 1 metabotropic glutamate receptor
mGluR4	Truncated type 4 metabotropic glutamate receptor
MOE	Main olfactory epithelium
MSG	Monosodium glutamate
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NEAA	Non-essential amino acids
NFAT	Nuclear factor of activated T cells
NPY	Neuropeptide Y
NST	Nucleus of the solitary tract
OR	Olfactory receptors
OSNs	Olfactory sensory neurons
OXM	Oxyntomodulin
P	Preference values
PLCβ2	Phospholipase C β2
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
pTas1r1	porcine taste receptor family 1 member 1 gene
pTas1r3	porcine taste receptor family 1 member 3 gene
pTas1r1/pTas1r3	porcine umami heterodimer gene
Q	Bitter
RAp	Rate of appetence
RR	Response ratio
RT-PCR	Reverse transcription polymerase chain reaction.
S	Sweet
SAA	Low crude protein diet supplemented with synthetic amino acids
SCCs	Solitary chemosensory cells
SGLT1	Glucose transporter isoform 1
T1R	Taste receptor family 1
T1R1	Taste receptor family 1 member 1
T1R2	Taste receptor family 1 member 2

T1R3	Taste receptor family 1 member 3
T1R1/T1R3	Umami taste heterodimer receptor
T1R2/T1R3	Sweet taste heterodimer receptor
T2Rs	Taste receptor family 2
TBP	Tata Box binding Protein gene
TC	Total consumption
TCC	Taste chemosensing cells
TL	Time of latency
TR	Taste receptors
TRC	Taste receptor cells
TRP	Transient receptor potential
TRPM5	Transient receptor potential M5 ion channel
Tα	Transducin
V	Vocalizations
VIP	Vasoactive intestinal polypeptide
VR-1	Vanilloid receptor-1
α-MSH	Melanocyte stimulating hormone

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Summary

Summary >>>>>>>>

The present work was divided into three main areas of study, (1) the development of a reliable "*in vivo*" model for double choice (DOCH) testing in piglets avoiding the isolation time and fasted state of animals to evaluate weaned pig preferences, consumption and appetence under a fed-state for Glycine and several L-amino acids at different concentrations (-*in vivo* trials- Chapter 1), (2) identifying and characterizing the porcine T*as*1r1 and T*as*1r3 gene sequences in pigs, to construct a stable cell reporter system expressing the porcine umami taste receptor (pT1r3/pT1r1) to measure the cell responses to amino acids at physiological concentrations (0.5, 5 and 50mM) and to compare these cell response with the pig *in vivo* results (-*in vitro* trials- Chapter 2), and (3) to determine the pattern of expression of the porcine umami taste receptor genes, pT*as*1r3 and pT*as*1r1, in several tissues of the digestive system including tongue (circumvallate and fungiform papillae), stomach (fundus), pancreas, liver, duodenum, jejunum and ileum and how that pattern responds to three factors: (a) the age of the pig -from birth to 20 days after weaning-; (b) sex –male vs. female-; and (c) the dietary crude protein content and essential amino acid supplementation (Chapter 3).

Chapter 1 describes a new methodology of DOCH testing to evaluate preferences, consumption and appetence in fed pigs for amino acid solutions (D,L-Met, Gly, L-Ala, L-Gln, L-Glu, L-Lys, L-Thre, L-Trp and MSG) at different concentrations (0.5, 5, 50 and 500mM). This methodology includes a preliminary training period (10-minute at 9am and 12pm) based on an operant conditioning procedure using sucrose at 500mM as a reward before test sessions (2-minute at 9am and 12pm). Two models were developed where animals were maintained individually (individual model) or in pairs (pair model) during training (4 and 2 days, respectively) and test sessions. Social isolation and novelty are two important factors of stress that could influence pig learning capacity for DOCH testing, therefore, behavioural and total test consumption parameters were used as criteria for the exclusion of animals in testing sessions. The results showed that the pair model developed in this work may be more

appropriate than previous models to study pig preferences and appetence for amino acid solutions or other nutrients. Weaned pigs under a fed-status were able to discriminate solutions of amino acids except when those were offered at 0.5mM. In general, piglets showed significant preferences for non-essential amino acids with a higher appetence for potential umami tastants at high concentrations (MSG, L-Glu and L-Gln). However, this taste response changed with essential amino acids, resulting in significant aversions at high concentrations (L-Trp, L-Thr).

Chapter 2 presents, after the identification and characterization of the porcine umami taste receptor (pT1r1/pT1r3), an *in vitro* tool based on a cell culture that expresses the heterodimer to identify Gly and L-amino acids as umami tastants at different concentrations (0.5, 5 and 50mM) in pigs. This cell system showed significant responses to MSG, L-Glu, L-Gln, L-Ala, L-Asn and Gly at all tested concentrations. Moreover, our *in vivo* data (Chapter 1) was significantly correlated with our in vitro results meaning that umami agonists are highly preferred by pigs.

Chapter 3 studies the expression of the porcine umami taste receptor genes, pT*as*1r1 and pT*as*1r3, in different taste (tongue's fungiform and circumvallate papillae) and non-taste tissues from the gastrointestinal tract (stomach, duodenum, jejunum, ileum and liver) of pigs of different sex (male and female) and ages (birth, preweaning- 26d old-, 48h after weaning -28d old- and 20d postweaning -46d old-) and with different levels of dietary crude protein in their postweaning diet (high-crude protein -26%, HCP-, low crude protein-17%- with –SAA- and without –LCP- essential amino acid supplementation). Both genes were more expressed in tongue and stomach, followed by small intestine and liver. Significant changes in gene expression were observed with age, sex and dietary crude protein content and the main changes occurred after weaning, with more expression in males than in females and in the LCP group that in the other two groups.

It is concluded that pigs sense some amino acids as umami tastants that generate a pleasant stimulus. The umami tastants are sensed through the heterodimer receptor pT1r1/pT1r3, which is expressed in taste buds in the tongue and in non-taste tissues along the gastrointestinal tract. Changes in pT*as*1r1/pT*as*1r3 gene expression may reflect the nutritional status of the animal and a better understanding of the mechanism will help to develop new strategies (such as the use of umami ligands to stimulate their voluntary feed intake) for improving the adaptation of piglets to the postweaning period.

Literature Review

Literature Review >>>>>>>>

The weaning period in pigs

The weanling process; stress factors and consequences

Weanling is a natural process in mammalian development defined by the end of the period of suckling milk from the mother and the start of nourishing from other food sources. In the wild piglets undergo a long and progressive weaning process that occurs between the 9th and the 22nd week of age. In contrast, under current pig husbandry practices, piglets are weaned abruptly at the age of 3-4 weeks in Europe or even younger in the USA (Worobec et al., 1999; Mormède and Hay, 2003; Bolhuis et al., 2009). The objective of early weaning is to maximize the productivity of sows and the utilization of the farrowing facilities (Maxwell and Carter, 2001). Nevertheless, young pigs have immature immune and digestive systems. Thus, the earlier the weaning the more acute the stress will be and more severe the consequences (Mormède and Hay, 2003).

The weaning period is regarded as one of the most critical periods in pig production, the level of feed intake and growth at weaning have an important impact in the subsequent future performance (King and Pluske, 2003). It is a well-known period of intense stress for the young pig with important consequences that affect its behaviour, immunity and performance. In fact, weaning is often associated with gastrointestinal disorders and a low growth rate due to insufficient voluntary feed intake (Maxwell and Carter, 2001). Some piglets refrain for eating for over 50h after weaning (Bruininx et al., 2002; Brooks and Tsourgiannis, 2003). The low level of feed intake just after weaning is the main factor that determines loses in the intestinal integrity, reducing the capacity of nutrient absorption and increasing the risk of gastrointestinal disorders, like proliferation of pathogens in the intestine due to an overload of undigested and unabsorbed nutrients (King and Pluske, 2003). Factors of stress in

piglets at weaning include the separation from the dam, reallocation involving mixing with unfamiliar piglets, introduction in a novel environment and eventually transportation to another place, radical dietary change (from milk to solid feed and water), and other changes in the environment (feeding and drinking equipment, temperature, humidity, ventilation, …).

Piglets need 3-4 days after weaning to reach the level of feed intake necessary to meet the energy requirements for maintainance (Le Dividich and Sève, 2001). Recovery from the weaning stress depends on weaning age and diet composition. For example, it has been showed that piglets weaned at 21 days need 4 days (McCraken et al., 1999) whereas younger piglets (7-14 days of age at weaning) may need up to 2 weeks for the recovery of the intestinal mucosa integrity (Marion et al., 2002). On the other hand, piglets weaned on to soybean meal-based diets suffer more intestinal damage and their growth rate is lower than those weaned on to animal protein (such as skim milk or fish meal)-based diets (Maxwell and Carter, 2001).

The importance of feeding behaviour in piglets after weaning

Pre-weaned piglets have little control over its food intake because the sow procures them nourishment. When weaning occurs gradually they learn about feed and water sources without any interruption of their milk nutrient supply. Under commercial conditions, sows have been selected to have more piglets. The maximum yield of milk in sows occurs at about four weeks and production falls gradually thereafter, although it varies considerably depending on genotype, parity, dietary regimen, body condition or litter size (Pettigrew, 1995). The total milk yield of a lactating sow increases linearly with the litter size, although yield per piglet decreases with an increase in litter size (Auldist and King, 1995; McDonald et al., 2002). The amount of milk produced by the sow and its composition limit the growth of the pig (Pluske et al., 1995). Harrell et al. (1993) calculated that milk production becomes limiting to the sucking piglet at around 8-10 days of age, increasing the difference between piglet requirements and sow's milk supply as lactation proceeds.

The radical dietary change at weaning contributes to cause a lack of appetite that may translate in anorexia and dehydration at weaning. Weanling piglets also need to learn to distinguish between hunger and thirst. They have to be able to satisfy their requirements by recognition of these two different sources: feed and drinking water (in exchange of milk that fulfills both requirements). Similar to the pre-weaning period, there are also wide variations in feed and water intake just after weaning and

particularly in the interval from weaning to the first feed. Almost 45% of piglets fast during the first 15h, and still 10% remain for more than 40 hours after weaning (Bruinix et al., 2001). Therefore, exposure to solid feed and water before weaning could be advantageous in order to improve feed intake, performance, immunity and welfare.

Prolonged starvation or even low level of feed and water intake during the first days after weaning results in piglets that do not cover their maintenance requirements. These animals loose body weight, suffer from gastrointestinal or other health disorders (mainly diarrhea), and have behavioural problems that could result in the death of the animal (Pluske et al., 1997). About 30% of the variation in "age to market" can be attributed to the time it takes a pig to reach a body weight of 25Kg (Fraser et al., 1995). Ilsley et al. (2003) reported that the weaning weight and growth during the initial post-weaning period is more important than birth weight and growth during the suckling period for predicting pig performance to slaughter.

Furthermore, weaning stress is known to decrease the digestive and absorption functions in different ways. The inadequate feed and nutrient intake at weaning results in gut villous atrophy, decreased enterocyte differentiation, reduction in villous crypt depth, suppression of pancreatic proteases and gut peptide secretions and increase in inflammatory processes (Cranwell, 1995; Miller and Slade, 2003). As a consequence, the malabsorption syndrome may appear, with ileal and colonic presence of unabsorbed nutrients and secretion of anorexigenic Glucagon-like peptide 1 (GLP-1) that further reduces feed intake (Pluske et al., 1997; Miller and Slade, 2003). In addition, weaning induces systemic effects through changing whole body nutrient metabolism, these changes are especially focused on circulating hormones such as insulin, glucagon and pro-inflammatory cytokines (McCraken et al., 1999) that in turn increase the amino acid catabolism and pig requirements (Lackeyram, 2003). Recent evidence shows that some amino acids are able to regulate intracellular protein synthesis and degradation, they are substrates for the synthesis of biologically active substances, such as nucleic acids, hormones and neurotransmitters, and enhance the immune function (Kim et al., 2007). For example, glutamine is a non-essential amino acid involved in intracellular protein metabolism that prevents intestinal atrophy (Wu et al., 1996), reduces glucocorticoid production in weanling pigs (Zhou et al., 2006) and enhances the immune status by increasing the phagocytic activity of macrophagues, T-lymphocytes cytokine production and B-lymphocytes antibody generation (Field et al., 2002).

Dietary strategies to reduce weaning stress in piglets

One of the most important priorities in piglet nutrition is to prevent weaning anorexia. Dry matter intake does not recover to the preweaning level until the second week after weaning (Brooks and Tsourgiannis, 2003). An optimal nutrition is more than the supply of adequate amounts of protein, energy, vitamins and minerals. To reach feeding requirements of piglets, the selection of ingredients should aim at a proper nutritional and digestible composition and also to preferred taste cues to obtain a very palatable (highly preferred) diet. Providing easily accessible drinking water fixtures and unlimited water supply should guarantee an adequate water intake. Eating and drinking behaviours are linearly associated in weaned pigs (Thacker, 1999; Tokach et al., 2003; Dybkjær et al., 2006). According to Dybkjær et al. (2006), attempts to increase feed intake before and after weaning should therefore include ways to optimize water intake in order to establish a balance between feed and water intake as soon as possible after weaning. Early exposure to palatable creep feed diets may help to improve the development of the structure of the gastrointestinal (GI) tract and the activity of digestive enzymes. Creep feed is often offered during lactation as a strategy to maximize piglet's growth, but its consumption is very low and there are wide variations in feed intake before weaning (Pluske et al., 1995). Pigs with the lightest body weight or that suckle the less productive teats, or have a lower average daily gain, tended to consume more creep feed than their littermates (Algers et al., 1990; Fraser et al., 1994), although those occupying the more productive teats also consume more creep feed than average piglets (BØe and Jensen, 1995). The benefit of creep feed exposure to lactating piglets is age dependent. Piglets weaned at 21 days or less show a low level of pre-weaning feed intake, insufficient to become familiar to solid feed, whereas litters weaned at 28 or more days may benefit more from pre-weaning creep feed due to their greater developmental maturity at weaning (Brunnix et al., 2002; Brooks and Tsourgiannis, 2003).

Piglets show neophobia in front of a novel feed. Again, the main strategy to improve feed acceptance may be increasing the palatability and digestibility of the diet. Recently, Bolhuis et al. (2009) indicated that offering a diet at weaning with similar flavour to their mother's gestation and lactation diets may reduce feed neophobia and stress in piglets, however, this strategy have difficulties to be implemented in current commercial conditions. It is also important the way in which the animals are fed (phases or choice-feeding). Animals that have the opportunity to choice-feeding are able to regulate their nutrient ingestion through the identification of nutrient or toxicant taste cues presents on a diet (Ettle and Roth, 2004; Manteca et al., 2008). Moreover, double choice fed pigs maintain a high-health-status minimizing immune

challenge which limits cytokine production and increases feed intake (Tokach et al., 2003). Feeders (design, space) and social factors may also affect feeding behaviour (Manteca et al., 2008; Manteca and Edwards, 2009). In addition to the individual learning process through sampling feed and its ingestive consequences, there is a role of social information transmission (from their mother -vertical transmission- or from their conspecifics) in the ontogeny of feeding behaviour (Bolhuis et al, 2009).

Weaned piglets lack the capacity to adapt their feed and water intake to their immediate nutritional requirements (Miller and Slade, 2003), but they have sensorial mechanisms to identify and to make a choice among feeds that differ in their composition or nutritional content (Ettle and Roth, 2004a; Roth et al., 2006; Solà-Oriol, 2008; Roura and Tedo, 2009). Taking into account that the digestive and immune system of weanling piglets are immature, they can easily be upset by inadequate nutrient supply such as amino acids. Diet composition has a high impact in pig adaptation to weaning. Some of the principal physiological factors limiting post-weaning growth in piglets are the inadequate level of digestive enzymes and acid secretion, the reduced absorptive capacity due to changes in villous structure, the lack of the beneficial bioactive compounds present in sow's milk (such as glutamine), the inadequate feed and water intake and the diet antigenicity (Partridge and Gill, 1993; Thacker, 1999).

A major concern when formulating transition diets is to provide adequate and balanced amounts of available amino acids to maximize protein deposition, especially in low body weight piglets. These diets should be formulated on amino acid to calorie ratios, rather than diet percentages, because weaned pigs are in an energy dependent phase of growth too (Tockach et al., 2003). When feed intake is no longer a critical factor, diet composition progressively changes looking for the lowest cost diets. Piglet's weaning feeding programs are structured in phases that try to balance pig performance and feed costs (Thacker, 1999; Tokach et al., 2003). In practical conditions, the nutritional value of a protein source depends on its amino acid composition, especially referred to their essential amino acid content and balance. According to Forbes (2009), the importance of "aminostatic" mechanisms in the control of food choice is clearly demonstrated by many choice-feeding studies in which growing pigs chose to eat proportions of high and low-protein foods to meet appropriate Protein:Energy ratios and sufficient to support close to maximal growth of lean tissue. On the other hand, the increase of post-weaning diarrhea in pigs due to the progressive ban of antibiotic use in animal nutrition has promoted the use of alternative management and nutritional strategies, such as reducing dietary crude protein content (Le Bellego and Noblet, 2002). Animals reject diets that lead to

indispensable amino acid depletion or deficiency by reducing feed intake. In turn, alleviation of essential amino acid deficiencies, through the diet or by infusion into the animal, increases feed intake to normal levels (Gietzen et al., 2007; Forbes, 2009). The supplementation of transition diets with crystalline amino acids, such as L-Lysine, D,L-Methionine, L-Threonine and L-Tryptophan is a common practice to guarantee the adequate essential amino acid supply when dietary crude protein content is reduced.

Sensorial perception in pigs, has evolved to help discriminate between beneficial (nutritious) and detrimental (toxic) compounds in feed resulting in preference or aversion, respectively (Goff and Klee, 2006). Newly weaned piglets depend mostly on its sensorial capacity and previous experience (social learning) to assess the nutritional or toxicant content of novel feeds; therefore, feed's sensorial characteristics play an important role at this stage (Forbes, 1995; Bolhuis et al., 2009). Use of highly preferred feed ingredients and nutrients may be an important strategy to stimulate feed intake in this critical period. Solà-Oriol et al. (2007) studied pig preferences for several feed ingredients and observed that relative to their dietary inclusion rates protein sources had a higher impact in preference than cereals. Tinti et al. (2000) showed preferences and aversions for L-amino acids in pre-fattening pigs and compared them to the human sensing without knowing the nature or the mechanism that triggered the taste perception in pigs. In humans and rodents, umami taste provides information about the L-amino acid and peptide content of the diet and is considered a taste cue that helps determining feeding behaviour (Roura et al., 2008). The study of the umami taste in pigs may be useful to identify protein derived nutrients that stimulate voluntary feed intake in weaned piglets and to better understand the effect of dietary proteins in pig physiology and metabolism.

Feed appetence in pigs

The concept of palatability

According to Roura and Tedó (2009), palatability is the perception of the quality of the food present in the oral cavity through oronasal sensing mechanisms. The perception is related to the food characteristics (quantity and nature of volatile –i.e. odorants- and non-volatile –i.e. tastants- compounds released, nutrient density, physical features such as texture, particle size and water holding capacity among others) but also to the physiological and genetic capacity of the individual and its metabolic status.

Recently, Solà-Oriol (2008) evaluated feed ingredient preferences in piglet diets and showed that the nature of the feedstuffs included in a diet affected feed palatability and voluntary feed intake. Oro-sensorial perception of feedstuffs, such as odour and taste, has evolved to trigger preference for nutritious or aversion for toxic compounds (Goff and Klee, 2006).

Ermer et al. (1994) defined that feed intake is the product of the number and size of meals consumed (meal patterns) and diet palatability is among the factors known to affect meal patterns. In rats, increasing palatability increases both, rate of feed consumption and meal size (Berridge et al., 1981; Le Magnen, 1985). Food palatability and hedonic value play central roles in nutrient intake. However, post-ingestive effects can influence food preference independently of palatability (Uematsu et al., 2009). Recent findings suggest that calorie-rich nutrients can directly influence brain reward circuits that control feed intake independently of palatability or functional taste transduction (De Araujo et al., 2008). However, it is necessary that the animal starts eating before post-ingestive effects may initiate. Palatability, in the sense of stimulus that trigger meal initiation, may be an important aspect of feed formulation to improve voluntary feed intake particularly during critical periods such as at weaning and at dietary changes.

Dietary preferences and voluntary feed intake

According to Forbes (2009), animals, including pigs, learn to associate the sensory properties of foods with internal signals generated after consumption and use this learned information as a key part of determining intake of foods and selection between them. Omnivores have developed preferences or aversions for some nutritional or toxic compounds identified as innate (Bolhuis et al., 2009). There is innate predisposition to prefer or reject basic tastes that are modified by experiences with food and eating (Birch, 1999). There is evidence for genetically coded individual differences in bitter taste perception. Many of the innate based feeding behaviours will be determined by the taste and smell perception mechanisms, some of which seem to be quite universally conserved across species, and known to be linked to nutritious compounds in humans and rodents (Roura et al., 2008). However, animals usually react with caution in front of novel feed sources. Behavioural studies prove that animals tend to show a neophobic reaction in front of feed novelties (Millman and Duncan, 2001). Neophobia reduces the risk of poisoning from unfamiliar feeds without completely limiting the exposure to them and is overcome only after post-ingestive and post-absorptive feed back signals guarantee innocuousness and nutritional balance (Roura et al., 2008). The learning process is important to change an initial neophobic rejection for a novel feed into a preference (Provenza and Balph, 1987). Fearfulness may also affect feeding behaviour and the acceptance of new foods.

Even within uniform groups of animals, individuals vary morphologically and physiologically and they also manifest differences in their diet selection and dietary breadth (Villalba et al., 2009). In contrast to learned food aversions, learned food preferences form more slowly and are more readily extinguished. Because learned preferences are a result of the more common consequences of normal eating, not as a result of pairing of food with illness, they may have more pervasive but subtler effects on food intake than do learned aversions (Birk, 1999; Bolhuis et al., 2009).

Voluntary feed intake of pigs determines nutrient intake levels and has a great impact on efficiency of pork production (Zijlstra and Scott, 2000). Food selection results from the balance among taste, smell and texture perceptions and other factors, including nutritional status, physiology and environment (García-Bailo et al., 2008). Pigs seem to adapt their feed intake to nutrient requirements, health and physiological status and genetic characteristics (Bruininx et al., 2001). Newly weaned piglets are not capable of adjusting intake based on nutrient digestibility and metabolism, but they are able to differentiate between different feeds through sensorial perception. Animals learn to associate the sensory properties of the feed with postingestional consequences and this experience becomes an important factor in the control of intake and diet selection (Forbes, 2009). For example, according to Black et al. (1986) pigs show the ability to adjust feed intake based on dietary energy concentration when they reach 20 to 30Kg of BW. Sugar appetite in pigs was reduced after gastric or intraduodenal injection of glucose, demonstrating that gastrointestinal factors are important in regulating intake (Baldwin, 1996). Taste perception provides information about the nutritional content of a diet. It has been shown that pig preferences for feedstuffs are related to the capacity of the ingredients to stimulate umami, sweet and salty taste in absence of bitter compounds (Roura et al., 2008).

Preference and aversion are behavioural expressions resulting from the integration of multiple sensory, metabolic and physiological inputs (Roura et al., 2008; Solà-Oriol, 2008). In addition to taste, other sensory systems, like olfaction, influence the animal predisposition to eat or reject a novel feed. Olfaction is organized to identify foods more holistically, rather than to identify nutrients, and to be readily influenced by learning and experience (Birch, 1999). The study of preferences (or aversions) for any nutrient or feed ingredient may be related to its nutritional and immunological value, and the animal previous experience (Van Heugten, 2001; Myers et al., 2005). Dietary preferences in pigs can be studied by choice-feeding trials (Solà-Oriol et al., 2008) where the animal chooses based on the qualities or the hedonic perception of feed and feed ingredients (Delay et al., 2007). Double-choice studies in pigs comparing different levels of a single nutrient in a diet or diet complexity, have assumed that the animal will select a feed combination that

maximizes its growth and performance because of meeting its nutritional requirements (Kyriazakis and Emmans, 1995; Morgan et al., 2003; Ettle and Roth, 2004a). Several studies have reported that feed intake and growth increases when animals are fed complex compared to simple diets (Mavromichalis et al., 2001; Wolter et al., 2003). In addition to choice-feeding trials, electrophysiological recordings from cranial taste nerves (Chorda Tympani –CT- and Glossopharyngeal -GP-) have been used to complement behavioural tests (Danilova et al., 1999; Hellekant and Danilova, 1999; Li et al., 2005).

Sweet is a hedonic taste in pigs. The first work reported on sugar preferences showed increases in feed consumption after dietary inclusion of 3 or 5% of sucrose (Salmon-Legagneur and Fevrier 1956). Kennedy and Baldwin (1972) showed that ad libitum fed pigs maintained their preferences for different natural and synthetic sweetener solutions in long and short-term (12h and 1h respectively) double choice tests. In these studies, pigs responded to sucrose, glucose and sodium saccharin in water. The threshold ranged from 5 to 10mM for sucrose and 10 to 30mM for glucose. For both substances animals showed consistent preferences (higher than 90%). Results for sodium saccharin were more variable. The threshold preference was from 5 to 10mM although pigs never showed preferences higher than 90% and even between 100 to 1000mM animals started to reject the solution. Roura et al. (2008) suggested that evolution has resulted in variations in the sequences of taste receptors (TR) that do not affect the binding of natural carbohydrate sugars, but may affect the binding of artificial ligands. Electrophysiological recordings from CT and GP nerves exhibited large responses to carbohydrates such as sucrose, lactose, maltose, glucose and galactose among other mono and oligosaccharides (Danilova et al., 1999). Glaser et al. (2000) showed that these results were consistent with the preference values for sweeteners. Preference tests were carried out using a 1-minute double-choice test pig model adapted from the Richter two-bottle drinking model, which includes a previous training session (Kennedy and Baldwin, 1972). Sucrose showed the highest intensity of all the carbohydrates tested and their responses were very similar to sugar sensory tests in humans (Table 1). Furthermore, pigs seem to be responsive to most D-AA (Table 2) known to be sweet to humans. Glycine might have a complex taste for pigs that includes stimulation of putative umami, sweet, bitter and sour fibers (Danilova et al. 1999; Hellekant and Danilova 1999). On the other hand only minor or no responses to other non-carbohydrate substances known to be sweet to humans have been reported in pigs (Danilova et al. 1999; Glaser et al. 2000; Hellekant and Danilova 1999; Tinti et al. 2000). For example, pigs did not respond to human-based doses of aspartame, neohesperidin, thaumatin or saccharin (Table 1), four well-known high intensity sweetners (HIS) in humans (Glaser et al. 2000; Matsunami et al. 2000). Sodium saccharin, a sweetener widely used in pig diets, elicited a response only three times above that of sucrose (Glaser et al. 2000).

Table 1 > Sweetener potencies relative to sucrose (on a molar basis) in humans and pigs (from Roura and Tedo, 2009[1]).

SWEET TASTANTS		HUMANS	PIGS[2]
Carbohydrates	Sucrose	1.00	1.00
	D-fructose	0.50	0.50
	Lactose	0.33	0.15
	D/l-glucose	0.25	0.13
Non-Carbohydrates	Aspartame	155	< 1.00[3]
	Cyclamate (Na^{+} salt)	17.6	< 0.15[3]
	Nehoesperidin (dihydrocalcone)	3.600	< 151[3]
	Thaumatin	2.000	< 1,622[3]
	Saccharin	215	3.34

1 - Adapted from Glaser et al. 2000. / 2 - Sweetener potencies in pigs were measured based on double choice preference tests.
3 - Equivalent to the lowest dose tested that gave no response. The exact potency was not determined.

Umami is also a hedonic taste in pigs. Umami compounds such as monosodium glutamate (MSG) and L-amino acids are frequently used as feed additives in pigs. Similar to sweet, double-choice trials and electrophysiological nerve recordings demonstrated that umami compounds stimulate taste in pigs (Danilova et al., 1999; Hellekant and Danilova, 1999; Glaser et al., 2000; Tinti et al., 2000). Tinti et al. (2000) employed the same double-choice test model than Glaser et al. (2000) to assess gustatory responses to amino acids in pigs and compared them to humans. Except for the D-amino acids, Glycine (Gly), L-Alanine (L-Ala), L-Glutamine (L-Gln), L-Hydroxiproline (L-Hyp), L-Serine (L-Ser), L-Asparragine (L-Asn) and L-Threonine (L-Thr) showed the highest preference in pigs. Four of these amino acids (L-Ala, L-Ser, L-Thr and Gly) were described as sweet in humans (Table 2). Data from rats showed that L-Ser and Gly were perceived differently when they were compared to MSG on a molar basis (Delay et al., 2007). Nerve recordings showed that gustatory responses to L-amino acids are considerably potentiated by purine nucleotides such as inosine monophosphate (IMP) (Yoshii et al., 1986). Pigs offered dietary choices varying in their protein level chose appropriate ratios to meet their nutritional requirements (Kyriazakis et al., 1990; Bradford and Gous, 1991). Taste cues seem to be relevant in several studies about pigs preferences of diets varying in different amino acid contents such as L-Trp, D,L-Met, L-Lys or L-Thr (Kirchgessner et al., 1999; Ettle and Roth, 2004; Roth et al., 2006).

Pigs, such as humans, detect and avoid bitter compounds (Nelson and Sanregret, 1997). Bitter taste has been related to identification of naturally occurring compounds in feedstuffs that may pose a threat for the animal such as anti-nutritional factors (ANF), poisons, drugs or toxicants. The fundamental principle of peripheral bitter sensing is wired to mediate the behavioural aversions to feed. Bitter taste is linked to a bigger family of taste

receptors, the T2Rs. Farm animals apparently respond more vigorously to bitter and feed aversions than to preferences (Roura et al. 2008). A 48-h double-choice feed preference model in pigs showed statistically significant aversions to feed already at 24h from the start while positive preferences do not become significant before 48h (Tedo et al., 2007).

Pigs have a high sensitivity for sour taste elicited by acids. Danilova et al. (1999) found citric but also ascorbic acid to elicit the largest CT electrophysiological responses of all the 30 potential tested tastants. Ettle et al. (2004b) studied the effect of acidifying diets with potassium diformate (K-diformate), formic or sorbic acid on dietary preferences in piglets. Animals chose unacidified diets when acidified diets included organic acids, presumably because of negative taste cues, although when these animals could not choose, they did not reduce their feed intake. Acidifiers are commonly used in weaner diets because of improving intestinal health and performance thanks to a reduction on the acid binding capacity of feeds that decreases bacteria proliferation, particularly pathogens (Gabert and Sauer, 1994; Roth and Kirchgessner 1998; Partanen 2001). Pig also responds to salty tastants, such as to chloride salts (Danilova et al. 1999). Weanling pigs are known to respond to the addition of sodium chloride to complete diets that are relatively high in sodium (Mahan et al. 1996).

Table 2 > Gustatory responsiveness of pigs to L and D isomers of amino acids and predominant hedonic response in humans (from Roura and Tedo, 2009[1]).

AMINO ACID	L-isomer		D-isomer	
	HUMAN TASTE[2]	PIG RESPONSE	HUMAN TASTE[2]	PIG RESPONSE
Alanine	Sweet	Yes	Sweet	Yes
Asparagine	Complex	Yes	Sweet	Yes
Glutamine	Complex	Yes	Sweet	Yes
Glycine[3]	-	-	Sweet	Yes
Histidine	Bitter	No	Sweet	No
Hydroxyproline	Sweet	Yes	Complex	No
Isoleucine	Bitter	No	Sweet	No
Leucine	Bitter	No	Sweet	No
Phenylalanine	Bitter	No	Sweet	Yes
Proline	Complex	No	Bitter	No
Serine	Sweet	Yes	Sweet	Yes
Threonine	Sweet	Yes	Sweet	Yes
Tryptophan	Bitter	No	Sweet	Yes
Valine	Bitter	No	Sweet	No

1 - Adapted from Tinti et al. 2000. / 2 - The predominant human taste was qualified by the authors in three categories: sweet, bitter or complex. Complex includes hedonic descriptions by the authors such as: flat, meaty, bitter and sweet. / 3 - Glycine has only one chiral.

Feedstuffs or nutrient preferences may be linked to its organoleptic characteristics, often indicative of their nutritional and health associated value (Solà-Oriol, 2008). Pigs under a fed-status may recognize these nutrients, which stimulate their voluntary feed intake (Ettle and Roth, 2004a, Roth et al., 2006). In pigs, positive taste stimulation is especially important when appetite is suppressed, such as at weaning, disease state or after social mixing, among others. Roura et al. (2008) hypothesized that increases of feed intake thanks to feed palatability could explain most of the improved performance parameters, particularly in young animals. Therefore, knowing ingredient or nutrient preferences should facilitate the initiation to feeding in piglets at weaning, and improve their performance and well-being.

The control of feed intake and appetite

To regulate feed intake, the brain must modulate appetite, and the core of appetite regulation lies in the gut-brain axis (Cummings and Overduin, 2007). Appetite, feed preferences and regulation of feed intake are key aspects of energy balance and homeostasis (Power and Schulkin, 2008). According to Wren and Bloom (2007), peripheral signals involved in regulation of body weight and ingestive behaviour are often categorized as long-acting adiposity signals such as insulin and leptin, and short-acting gastrointestinal factors. These adiposity hormones function as gain-setters to modulate the sensitivity of vagal and hindbrain responses to GI satiation signals and regulate short-term food intake to achieve long-term energy balance. Therefore, long acting signals characteristically reflect the levels of energy stores and regulate body weight and the amount of energy stored as fat over the long term. Short acting gastrointestinal signals are typified by gut hormones such as CCK and mechanical factors, such as gastric distension, which characteristically relay a sense of "fullness" (satiety) resulting in postprandial satiation and meal termination (Cummings and Overduin, 2007). Nutrients and their metabolites control short and long term feed intake through direct or indirect endocrine secretions that interact with local and central neural processes. The hormones act collectively to control meal size suppressing intake through jejunal, ileal and/or colonic brakes, considered as short-term control of GI tract function, but they also act through vagal innervations or directly to specific brain areas to have long-term effects on feed intake (Black et al., 2009; Carroll and Allee, 2009).

Taste interacts with the central nervous system (CNS) and the gastrointestinal (GI) tract integrating the response to physiological mechanisms that also occur in other tissues. Short and long-term control of feed intake and energy balance are regulated through distinct mechanisms that interact with each other, involving

peripheral and central systems (Chaudhari et al., 2008). Peripheral signals that regulate feed intake are hormones and peptides from the adipose tissue (Leptin, Adiponectin and Resistin), pancreas (Insulin, Enterostatin, Amylin, Pancreatic Polypeptide-PP-) and GI tract (PYY, Oxyntomodulin-OXM-, Ghrelin, Gastric leptin, Glucagon-like peptide 1 – GLP-1-, Cholecystokinin- CCK- and Bombesin) and are involved in the energy homeostasis (Stanley et al., 2005; Cummings and Overduin, 2007; Chaudhari et al., 2008; Chaptini and Peikin, 2008). Central signals for the regulation of appetite and energy balance, in turn, are orchestrated from the hypothalamus and brainstem. Several neurotransmitters or neuropeptides have been identified at that level such as orexin, urocortin, neuropeptide Y-NPY-, pro-opiomelanocortin –POMC- peptide products like –melanocyte stimulating hormone –α-MSH-, Agouti related protein – AgRP- and cocaine- and amphetamine-related transcript – CART- (Chaudhari et al., 2008; Chaptini and Peikin, 2008; Black et al., 2009; Carroll and Allee, 2009) (Table 3). Peripheral signals influence central circuits in the hypothalamus, brainstem and limbic system to modulate neuropeptide release and hence food intake and energy expenditure. For example, an animal under food restriction has low levels of circulating leptin and this circumstance activates orexigenic pathways mediated by NPY and AgRP neurons whereas in times of plenty, high plasma levels of leptin activates anorectic POMC and CART neurons (Bray, 2000; Stanley et al., 2005).

Table 3 > Peripheral and central signals for regulation of feed intake and appetite in mammals.

Control of Appetite	Peripheral (Hormones)		Central (Neurons[3] and Neurotransmitters)
Orexigenic (Stimulates feed intake)	Ghrelin		NPY
			AgRP
			Orexins
Anorectic (Inhibits feed intake)	GLP-1[1]	PYY	Urocortin
	OXM[1]	Insulin	POMC (α-MSH)
	PP[1]	Leptin	CART
	CCK[1]	Bombesin	CCK[2]

1 - Gastrointestinal Satiety Signals (Chaudhari et al., 2008 ; Chaptini and Peikin, 2008) / 2 - CCK is also expressed within the CNS, acting as a neurotransmitter (Stanley et al., 2005) / 3 - Populations of neurons that coexpress NPY, AgRP, POMC and CART(Chaudhari et al., 2008; Chaptini and Peikin, 2008).

At the central level, reciprocal connections exist between the hypothalamus and brainstem. The CNS centers of appetite control are: the area postrema (AP) in the brainstem and the median eminence (ME) in the hypothalamus. Both centers lack a complete blood-brain barrier, which means that they are susceptible to circulating factors (although this is controversial in the case of ME). In the brainstem, nearby the AP, the nucleus of the solitary tract (NTS) receives vagal afferent fibers from the GI tract and from the glossofaringeal and facial nerves involved in taste perception. In the hypothalamus, the arcuate nucleus (ARC) has been described as a site of integration of a number of neurological and blood-borne signals. In the ARC there are two major populations of neurons established that influence appetite (Table 3): the orexigenic (coexpressing NPY and AgRP) and the anorexigenic (coexpressing POMC and CART). The AgRP antagonizes the effects of the POMC product by inhibiting α-MSH (*Figure 1*). These populations of neurons are also projected to the hypothalamic paraventricular nucleus (PVN) involved in pituitary hormone secretion and appetite regulation (Stanley et al., 2005; Chaudhari et al., 2008; Roura and Tedo, 2009).

Two main pathways located in the hypothalamus, the AMPK (adenosine monophosphate-activated protein kinase) and the mTOR (mammalian target of rapamycin), are directly involved in the metabolic long-term control of voluntary feed intake that monitors nutritional status and energy expenditure and body composition. The AMPK increases under a depletion of the energy stores and reduces the production of Malonyl-CoA. The Malonyl-CoA appears to be one of the most important intermediate product in the control of feed intake able to stimulate feed intake and reduce energy expenditure through the activation of orexigenic peptides of the melanocortin system (AgRP and NPY) and the inhibition of the production of anorexigenic peptides (POMC, CART). The mTOR is the opposite pathway, which under a high level of energy status decreases intake and increases energy expenditure. The insulin and leptin are also controlling the activation of mTOR in the hypothalamus (Black et al., 2009).

Figure 1 > Central Nervous System centers of appetite control and the pathways by which circulating factors may influence appetite (adapted from Chaudhari et al., 2008).
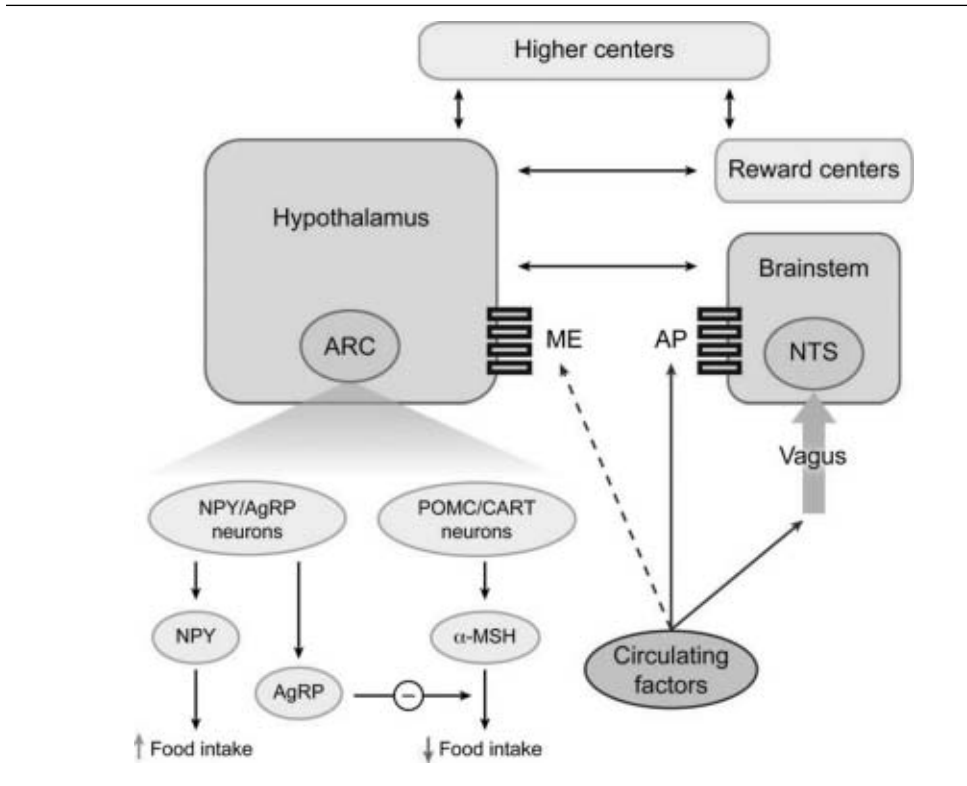
The area postrema (AP) in the brainstem and the median eminence (ME, denoted by the dashed line) in the hypothalamus. Nucleus of the tractus solitarius (NTS) in the brainstem. The arcuate nucleus of the hypothalamus (ARC). Subpopulations of neuron in the ARC, 1.- coexpressing neuropeptide Y (NPY) and agouti-related protein (AgRP), both orexigenic –stimulating food intake and 2.- coexpressing pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), both anorexigenic –inhibiting food intake. The AgRP antagonizes the effects of the POMC product, α-melanocyte-stimulating hormone (α-MSH).

The interaction between the nutritional status (fed and fasting states) and the sensation of reward (high palatability) may act as the stimulus to feeding when the energy requirements are covered. Stanley et al. (2005) described that the reward circuitry is complex due to the interaction among different signaling systems, like opioid (Enkephalin, β-endorphin), dopaminergic (Dopamine) and cannabinoid (Endocannabinoids) systems among others (GABAergic, Serotoninergic, Noradrenergic connections and Neurotensin). Opioids stimulate the preferential appetite for palatable substances like sugar or fat (Zhang et al., 2003), cannabinoids stimulate food intake in rats and humans (Abel, 1975; Koch, 2001),

and dopamine is also able to stimulate feeding and preference for a palatable diet (Szczypka et al., 2001).

The link between the GI tract and the brain is the vagus nerve, which acts as a mediator of efferent signals for the cephalic phase of digestion (Zafra et al., 2006) and its afferent signals convey information about food borne mechanical or chemical stimulation of the GI tract (Chaudhari et al., 2008). There are also vagal mechanosensitive fibers stimulated by gastric loads that are responsible for the meal termination, independently of the nutrient content of the load (Schwartz et al., 1991, 2000; Mathis et al., 1998).

Cephalic phase responses in the regulation of feed intake

According to Zafra et al. (2006) the cephalic phase of nutrition refers to a set of food intake-associated autonomic and endocrine responses to the stimulation of sensory systems mainly located in the oropharyngeal cavity. The cephalic phase response takes place in different parts of the digestive system mediated through efferent fibers from the vagus nerve. Other structures from the brain and other nerves have also been involved. The cephalic phase has been considered as an anticipatory physiological regulation and an adaptive strategy that serves to prepare the digestive tract to digest food and absorb nutrients, and to prepare other tissues, like liver and adipose tissue, to metabolize and store the absorbed nutrients (Power and Schulkin, 2008). But cephalic phase responses can also serve to inhibit feeding and prepare an animal to deal with toxic compounds present on a diet, for example, bitter taste compounds decrease gastric motility (Wicks et al., 2005).

The main responses during the cephalic phase are observed in the oral cavity (saliva), stomach (hydrochloric acid, gastrin, lipases, immunoglobulins and leptin), duodenum (bicarbonate), pancreas (bicarbonate, lipases, amylases, trypsin, chymotripsin, insulin, glucagon, PP, CCK), gallbladder (secretions) and adipose tissue (enzymes) among others. There are other responses that are non-secretory, like gastric emptying and gut motility, both regulating food passage, and thermogenesis, the increase in metabolism and body temperature induced by feeding may in part be an adaptation to defend homeostasis by metabolically removing glucose and fatty acids from circulation (Table 4). Cephalic phase responses may also play a role in appetite and satiety, and thus in the beginning and ending of a meal. For example, both ghrelin secreted by the stomach (Drazen et al., 2006) and insulin secreted by the pancreas (Just el al., 2008) appear to exhibit a

cephalic phase. Insulin early increases its secretion following the taste stimulation (within 4 minutes).

Cephalic phase responses can be demonstrated using the technique of sham feeding (Power and Schulkin, 2008). Sham feeding consists in using fistulas at different levels of the GI tract (esophagus or stomach), allowing mastication of the food and swallowing but without letting the food arrive to the intestines. Sham feeding stimulates secretions of pancreas and the stomach that results in changes in the GI tract, blood stream and in behaviour. In humans, dogs and rats it has been demonstrated that food palatability has a positive correlation with the extent and magnitude of the cephalic salivary and gastric secretions (Pavlov, 1902; Goldschmidt et al., 1990; Martínez et al., 2002).

The digestive process starts when food is masticated and mixed with saliva, which is essential for taste perception. Taste promotes or inhibits food intake and prepare the body to utilize or metabolically respond to ingested compounds. Through the cephalic phase responses, the sensory aspects of food interact with the metabolic state of individuals influencing their feeding behaviour (Power and Schulkin, 2008). The scheme of the events may be divided into different levels. There is an oropharyngeal phase, where food stimulation is essential to different nutrition-related processes like satiety, appetite and the development of preferences and aversions, and continues with the neural/cephalic phase, referred as a set of physiological, endocrine and autonomic responses of the digestive system that results from stimulation of sensory systems, mainly taste and olfaction (Zafra et al., 2006). These phases ensure an appropriate utilization of ingested foods for optimal nutritional use since they prepare the digestive system for food reception and control of the metabolic processes that allow nutrient absorption. (Zafra et al., 2006; Solà-Oriol, 2008).

Table 4 > Main cephalic phase responses (adapted from Zafra et al., 2006; Power and Schulkin, 2008).

LOCALIZATION	RESPONSE	ACTIONS AND MECHANISMS
Oral cavity	Saliva	· Essential for taste perception due to dissolution of food particles into the taste buds. · Lubricates and protects oral mucosa. · Starts digestion of nutrients
Stomach	1-Hydrochloric acid 2-Gastrin 3-Lipases 4-Immunoglobulins 5-Leptin 6-Pepsinogen	1-Breakdown of food 2-Gastric acid secretion 3-Breakdown of fats 4-Protection of gastric and gut mucosa from exogenous microorganisms from food. 5-Satiation through leptin receptors from vagal afferents. 6- Breakdown of protein
Duodenum	Bicarbonate	Duodenal neutralization of the acid from gastric emptying.
Pancreas	1-Bicarbonate 2-Digestive enzymes 3-Insulin 4-Glucagon 5-PP and CCK	1-Neutralization of duodenal acid contents. 2- Breakdown of fats, carbohydrates and protein. 3-Anticipatory metabolic role. 4-Prevention of hypoglycemia. 5-Biological role uncertain.
Others	1-Secretory : gallbladder flow, secretin and enzymes in adipose tissue 2-Non-secretory: gastric motor activity, gastric emptying, intestinal motor activity, postpandrial thermogenesis.	In general all of them help in digestion, absorption and utilization of ingested foods and also in the non-secretory, there is as an action in the regulation of food passage.

The short-term effects of cephalic phase responses may play a direct role in determining meal size and total daily food intake. Cephalic phase responses increase the efficiency in transforming food into nutrients, due to their ability to stimulate digestive processes, and absorbing them in the intestine. This allows a higher total food intake, shorter latency times between meals, shorter feeding time to meet nutritional requirements and higher nutrient absorption of foods with lower quality. At the same time, it is also required rapid and efficient metabolic responses to maintain the homeostatic levels of the subsequent absorbed nutrients. Palatable foods generally result in more robust cephalic phase responses than do less preferred food (Power and Schulkin, 2008). It may explain why these foods may be ingested in higher quantities.

Peripheral sensing in pigs

According to Roura and Tedó (2009), peripheral chemosensing functions as a decoding system that interprets among other stimuli those relevant to the nutritional value of feedstuffs. All five senses can influence preferences and feed intake in pigs, although not all of them at the same level of importance. Sight is not the most developed sense in the pig. Pigs posses dichromatic vision (able to discern only between short and long wavelengths) and do not differentiate colours (Edge et al., 2004). Sounds (hearing) may as well condition pig feeding behaviour. For example, auditory stimulation with nursing vocalizations stimulated feeding and drinking behaviour in newly weaned piglets (Kilgour, 1978, Petrie and Gonyou, 1988). Belonging to the somatosensorial perception, pigs are responsive to differences in feed and water temperature and feed texture (Reiners et al., 2008; Solà-Oriol et al., 2009).

Food ingestion simultaneously evokes odor, taste, and thermo-mechanical sensations that stimulate appetite for appropriate ingredients that ensure self-nourishment. Roura and Tedó (2009) described the peripheral nervous system connecting the oronasal cavity to the central nervous system (CNS, brain and spinal cord). The system consists of the nerves described in Figure 2: Olfactory (I cranial nerve-Olfaction), Trigeminal (V cranial nerve – Somatosensing), Chorda Tympany (from the VII cranial nerve or Facial nerve- Taste), Glossopharyngeal nerve (IX cranial nerve- Taste) and Vagus (XI cranial nerve- Taste).

Figure 2 > Cranial nerves of the peripheral nervous system in the pig (drawings by Joaquim Roura, 2009).

— Cranial nerve I (Olfactory)
— Cranial nerve VII (Chorda Tympany)
— Cranial nerve IX (Glossopharyngeal)
— Cranial nerve X (Vagus)

— Cranial nerve V (Trigeminal)
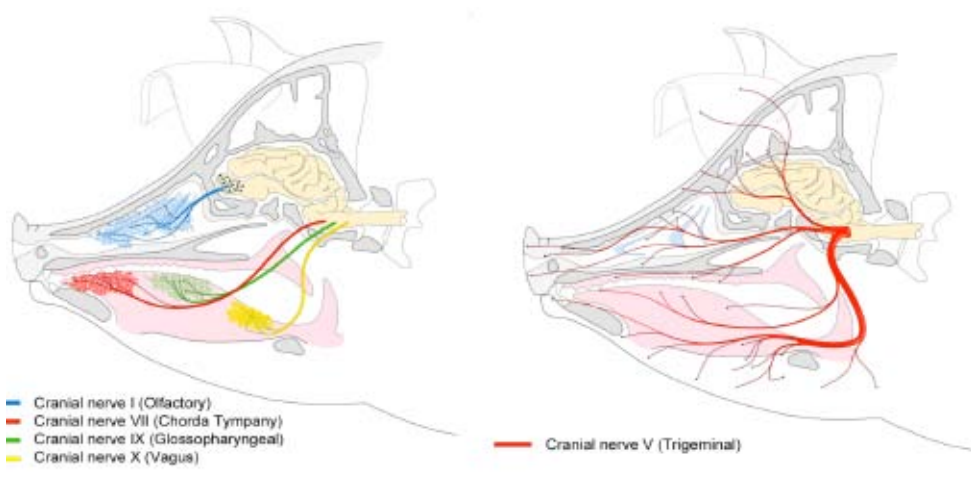
The three most important chemosensory systems that play a crucial role in nutrient intake are taste, smell, and somatosensing. This section will focus on describing the physiology of all of them (smell, somatosensing and taste) in the pig and their function in associating beneficial (nutritious) and detrimental (toxic) compounds with pleasant or unpleasant sensations.

Olfaction in pigs

The biology of olfaction

Olfactory perception has been classified in orthonasal (external air is inspired) and retronasal (air expired from the oral cavity), both perceptions may include interactions by other peripheral stimuli. Orthonasal perception integrates the olfactory pathway with the vomeronasal (pheromonal), sight, sound and touch (somatic) and the retronasal involves only food volatiles integrated with taste and oral somatosensing cues. Therefore, the perception of flavours while ingesting feed (retronasal) integrates at least these three chemosensory systems: olfaction, taste and somatosensing (Shepherd, 2006; Bolhuis et al., 2009; Roura et al., 2009). On the other hand, the olfactory system is characterized by several features that include: a high number of olfactory receptors (OR), multiple compounds might be recognized by each OR and each compound being able to bind to multiple OR. These combinatorial strategy of OR stimulations account for a very high number of odor identities recognizable by the mammalian olfactory system (Zhao et al., 1998; Malnic et al., 1999; Firestein, 2001; Rouquier and Giorgi 2007; Roura et al., 2008).

Olfaction occurs in the main olfactory epithelium (MOE) located in the upper wall of the nasal cavity. The MOE consists of turbinates that increase the surface area, a basement cell membrane and a stratified layer of supporting cells that contain the olfactory sensory neurons (OSNs) of the first cranial nerve (Olfactory nerve) (*Figure 2*). The OSNs are bipolar neurons with a single dendrite that reaches up to the surface of the neuroepithelium and ends in a knob-like swelling from which project some 20–30 cilia that lie in the superficial thin layer of mucus. OSNs are characterized by their ability to express olfactory receptors (ORs) on the plasma membrane of the cilia (*Figure 3*). The ORs are classified in two classes, class I related to fish-like receptors that bind water-soluble odorants, and class II receptors binding to air-born molecules. Ligand binding to OR transduces the chemical stimulus into an electrical stimulus through the olfactory bulb to the CNS (Firestein, 2001; Roura et al., 2009). The transmission of an odorant signal starts in the nose where several OR are excited. The

signal is transmitted through several neurons to the brain using the axons that reach the olfactory bulb. Those OSN expressing the same OR converge in the same glomeruli of the olfactory bulb that, therefore, is linked to a specific odorant. The axons of the mitral cells connect the glomeruli to the olfactory cortex where the information is organized and sent to other areas of the brain that will discriminate odors and trigger emotional and physiological effects (Buck, 2005; Roura et al., 2009).

Figure 3 > Scheme of the main olfactory epithelium and olfactory bulb (drawing by Joaquim Roura, 2009).

1 Nasal Lumen
2 Olfactory cilia
3 Mucus
4 Supporting cell
5 Olfactory sensory neuron
6 Basal cell
7 Basement membrane
8 Olfactory neuron axons
9 Glomerulus
10 Mitral Cell
11 Olfactory bulb
12 Olfactory Epithelium
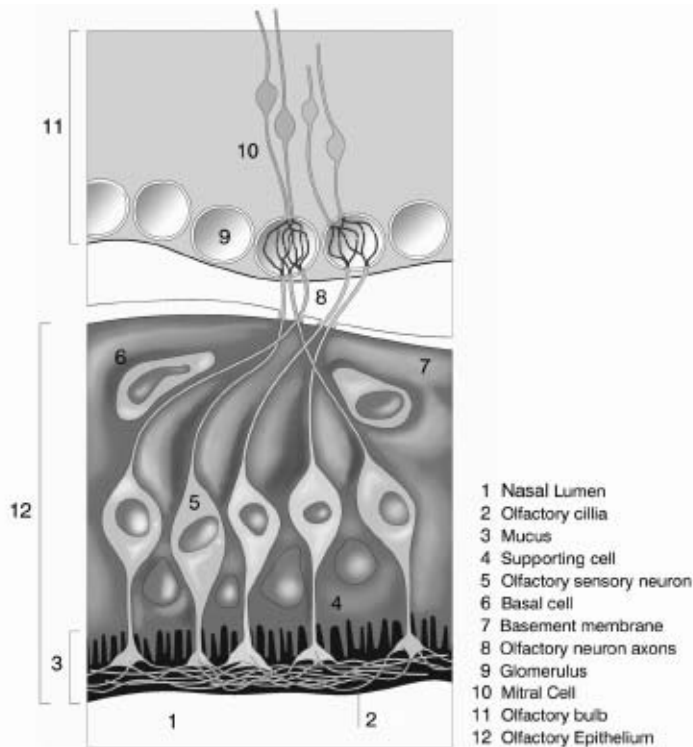
Olfaction and feed appetence in pigs

The sense of smell is present in most animal species and it emerges as the main sense after birth in pigs and most mammals (Morrow-Tesch and McGlone 1990). Mammals use volatile compounds (flavours), among other characteristics, to identify foods and their nutritional value. Some plant volatile compounds that stimulate feed consumption in animals have essential nutrients as precursors, like amino and fatty acids, whereas plant volatiles derived from anti-nutritional factors (ANFs) and

potentially toxicant compounds are rejected. According to Roura et al. (2008), the nutritional value of foods is species dependent. In pigs, olfactory sensitivity has been shown to be extremely high compared to primates and other animal species. Some evidences demonstrate that humans have less capacity to perceive odorants compared to pigs. Humans have a smaller olfactory epithelium surface, a lower number of OSN compared to pigs. Furthermore, humans have a high proportion of pseudogenized (inactive) OR (Roura and Tedó, 2009).

Piglets at birth are able to identify the odor from their mother and show significant preferences for odors associated with maternal feces and skin secretions (Morrow-Tesch and McGlone 1990). Other preferred smells in young pigs have been shown related to placental fluids and sow milk (Rohde Parfet and Gonyou 1991; Lay et al. 1999). Prenatal and early in life exposure to a flavour (concept that has been referred to as vertical transmission - Bolhuis et al., 2009-) may increase the animal acceptance to eat at weaning. The use of the same flavour in the weaner diets seems to reduce neophobia and stress, situation commonly observed at this stage. Bolhuis et al. (2009) described that the flavour conditioning of weanling pigs through vertical transmission increases feed intake and growth, reduces their stress and improves animal health and welfare. For example, it has been showed that aniseed and garlic flavours contain odorants that cross into both amniotic and allantoic fluids and milk allowing piglets to have a prenatal and early in life exposure. Litters from mothers with aniseed or garlic flavours in their diets (during gestation or/and lactation) had a higher feed intake compared to those of mother's without flavoured diets (Campbell, 1976; Hepper, 1988; Langendijk et al., 2007).

Double choice models have been widely used in determining flavour preferences in pigs. McLaughlin et al. (1983) studied several flavours with or without taste enhancers and showed preferences from buttery, green and meaty notes. Interestingly the majority of these flavours may be associated to nutrient volatile compounds. Solà-Oriol (2008) carried out several preference tests were showed that palatability of feed and feed ingredients may also depend on the odorants they release. For example, protein sources after storage obtained a lower preference in pigs than fresh because of the odorants that they released due to oxidation. Previously DeRouchey et al. (2004) showed that lipid oxidation may also compromise palatability. Therefore, olfaction is also critical in flavour perception and food preferences. Bolhuis et al. (2009) suggested that olfaction is organized to identify foods more holistically, rather than to identify nutrients, and to be readily influenced by learning and experience.

Somatosensing in pigs

The biology of the oronasal somatic sensing

According to Roura et al. (2008), oral-born somatic sensing has been defined as the non-taste and non-odorous sensations evoked by the presence of food in the mouth. Somatosensing includes thermal, mechanical and some chemical stimulations that are classified depending on viscosity (thin, thick and viscous), feel on soft tissue surfaces (smooth, pulpy and creamy), carbonation-related terms (bubbly, tingly and foamy), body-related terms (heavy, watery and light), chemical effect (astringent, burning, sharp), coating of oral cavity (mouth coating, clinging, fatty, oil), resistance to tongue movement (slimy, syrupy, pasty and sticky), after feel mouth (clean, drying and lingering), after feel physiological (refreshing, warming, thirst-quenching and filling), temperature related (cold or hot), and wetness related (wet or dry) (Szczesniak, 2002).

Somatic sensing of the oronasal cavity is driven mainly through two of the branches of the bilateral cranial nerve V (Trigeminal nerve) (*Figure 2*) (Witt et al. 2003), which is closely related to pain pathways (Tominaga and Julius 2000). Trigeminal sensory neurons project afferent fibers to various nuclei in the brainstem, not only in the trigeminal area such as trigeminal nucleus and principal nucleus, but also in the non-trigeminal area such the nucleus of the solitary tract, trigeminal motor nucleus, and reticular formation (Ohmoto et al., 2008). Information of noxious chemicals such as bitter compounds are transmitted to trigeminal neurons across a synapse, and further conveyed to the brainstem neurons, activating protective reflexes (Finger et al., 2003). Other commonly described noxious stimuli are thermal (low or high temperatures) and pungent (acids, spices) (Miller and Teates 1984; Hyde and Witherly 1993; Tominaga and Julius 2000). Those are perceived through the stimulation of transmembrane ion channel members of the transient receptor potential (TRP) family (Julius and Basbaum 2001; Dhaka et al. 2006; Caterina, 2007). The TRP's family includes the TRPV1 for detection of noxious hot temperatures (higher than 43ºC), the TRPM8 for detection of noxious cold temperatures (lower than 4ºC) (Dhaka et al. 2006) and the TRPA1 for detection of some noxious pungent chemicals (Caterina, 2007). Examples of chemical compounds that activate TRPV1, TRPM8 and TRPVA1 are capsaicin (hot sensation), menthol (cold sensation), and propionic acid, respectively (Caterina et al., 1997; McKemy et al., 2002; Caterina, 2007). These compounds that activate these TRP's receptors may damage the mucosa of the GI tract. Their activation leads to protective reflexes like feed rejection, stimulation of digestive secretions (salivary, gastric, bile, pancreatic and intestinal mucosa secretions) and an increase in the intestinal motility (Platel and Srinivasan 2004; Westendarp 2005; Jamroz et al. 2006).

Somatic sensing and feed appetence in pigs

Physical characteristics of feed and feed ingredients, like texture and particle size, have an impact on feed palatability through the stimulation of the somatosensing system (Roura and Tedo, 2009). Studies looking at the correlation between preferences and some physical characteristics of feed ingredients in pigs showed that hardness and chewing effort are negatively correlated with preference. Particle size is negatively correlated with preference in its extremes (the smallest and the biggest) whereas intermediate particle size had positive correlation with preference (Solà-Oriol et al., 2007b). Some chemical compounds, like botanicals and organic acids, can also stimulate the TRP's family. Several botanicals (herbs, spices and essential oils) and organic acids are commonly used as feed ingredients in pig diets, especially after banning feed antibiotic growth promoters in Europe. Their action may involve the activation of the oronasal somatosensing system that may reduce the voluntary feed intake in pigs through activation of pain pathways (Tominaga and Julius, 2000; Platel and Srinivasan, 2004; Srinivasan, 2007; Roura and Tedo, 2009).

Roura and Tedo (2009) suggested that there are no clear evidences that herbs, spices and essential oils improve palatability and feed intake in pigs. Their feed supplemented with moderate levels of cinnamaldehyde, carvacrol or capsicum oleoresin resulted in decreased feed intake (Bikker et al. 2003). Essential oils from fennel and caraway seed also decreased feed intake in weaned piglets (Schöne et al. 2003). The use of organic acids in pig diets showed that pigs preferred unacidified diets compared to acidified diets (Ettle and Roth, 2004b), probably because of the activation of the TRPA receptor that these authors suggested as negative taste cues. The inclusion of organic acids in pig diets improves intestinal health and performance thanks to a reduction on the acid binding capacity of feeds that decreases bacteria proliferation, particularly pathogens (Gabert and Sauer, 1994; Roth and Kirchgessner 1998; Partanen 2001). This positive effect may be the reason why when these animals could not choose, they did not reduce their feed intake (Ettle and Roth, 2004b). Although other studies in pigs showed that formic acid and combinations of formic and propionic acids decreased feed intake (Eisemann and van Heugten, 2007; Partanen et al., 2007). Sometimes the severe feed rejection or the positive effect may depend on the dose and/or the coating strategy for reducing pungent sensation. For example, moderate seasoning will stimulate digestive secretions (Platel and Srinivasam, 2004) whereas heavily spiced food (capsaicin) might trigger a pain-mediated alarm response that causes food rejection (Tominaga and Julius 2000). Pungent substances, like formic acid, absorbed in a carrier may improve the total feed intake compared to non-absorbed inclusions (Partanen et al., 2007).

Taste in pigs

The biology of taste

Taste is defined as the group of sensations mediated by the chemosensory system mainly located in the oral cavity (tongue, epiglottis and soft palate) that allows animals to identify nutrients and anti-nutritional compounds. Therefore, taste is a reliable and effective platform to help recognize and distinguish key dietary components that insure animals to choose food appropriate for their nutritional requirements (Holder, 1991; Dulac 2000; Nelson et al. 2002; Margolskee 2002; Chandrashekar et al., 2006; Bachmanov and Beauchamp 2007; Solà-Oriol, 2008; Roura et al., 2008). Humans and most non-primate mammals studied to date (except cats -Li et al., 2005-), share significant similarities in the nature and mechanisms of the five primary taste activities defined as sweet, bitter, sour, salty and umami. Sweet taste identifies carbohydrates (energy), umami recognizes amino acids (protein), salt taste targets proper dietary electrolyte balance, and sour and bitter warn against the intake of potentially noxious and/or poisonous chemicals (Chandrashekar et al., 2006; Boughter and Bachmanov, 2007; Bachmanov and Beauchamp 2007; Roura et al., 2008). Other candidate taste families are being studied such as a taste for complex carbohydrates (Sclafani, 2004), calcium (Conigrave et al., 2000; Tordoff, 2001; , Conigrave and Brown, 2006; Tordoff et al., 2008), hypo-osmotic fluids (Galindo-Cuspinera et al., 2006; Gilbertson et al., 2006) and lipids (Laugerette et al., 2005; Matsumara et al., 2007). Taste perception variability among animal species may contribute to understand species differences in nutritional and dietary requirements.

The detection of gustatory stimuli depends on chemosensory cells located in the taste buds of the oral cavity. Taste buds are primarily present in the tongue, mainly grouped in fungiform, foliate and circumvallate papillae, although are found as well in other areas of the digestive system as the soft palate, epiglottis, uvula, pharynx, larynx and esophagus (Lalonde and Eglitis, 1961; Gilbertson et al., 2000, Mombaerts, 2000). Every mouth papillae contains several thousands of taste buds and differs in its distribution among mammals. In humans, there is a positive correlation between the number of fungiform taste buds and the ability to taste; there is an inverse correlation between the fungiform papillae density and the detection threshold (Miller and Ready, 1990, Zhang et al., 2009). The pig differs from other mammals in the number of papillae and taste buds (Table 5). For example, pigs have more fungiform (5000 vs. 1600), circumvallate (more than 10000 vs. 6000) and foliate (4800 vs. 3000 foliate) papillae than humans (Miller, 1986; Chamorro et al, 1993; Hellekant and Danilova, 1999), suggesting that pigs have higher taste accuracy than humans.

Table 5 > Estimated number of taste buds in different mammals (adapted from Roura et al., 2008).

ANIMAL SPECIES	TASTE BUDS IN TONGUE	LITERATURE CITED
Dog	1706	Leibetseder, 1978
Cat	2755	Robinson and Winkles, 1990
Man	aprox. 5000	Miller, 1986
Pig	19904	Chamorro et al., 1993
Cow	aprox. 20000	Davies et al., 1979

In pigs, like other mammals, fungiform papillae are distributed on the tip of the tongue and are innervated by the facial branch of the cranial nerve VII (Chorda tympani, CT). Circumvallate and foliate papillae are on the back of the tongue and are both innervated by the cranial nerve IX (Glossopharingeal, GP) (Danilova et al., 1999; Witt et al., 2003). On the other hand, the superior laryngeal nerve, an afferent branch of the vagal (X) nerve, innervates the taste buds in the epiglottis and pharynx (Norgren, 1995; Gilbertson et al., 2000). These nerve's afferent fibers are responsible for the transmission of taste related peripheral stimuli (*Figure 2*) playing a role in determining taste discriminations and preference (Yasuo et al., 2008). In rats, electrophysiological and behavioural responses among CT, GP and greater superficial petrosal (GSP) nerves, the three major branches of cranial nerves innervating taste buds, suggested that umami taste is conveyed more dominantly via the CT and GSP than the GP (Sako et al., 2000).

Taste chemosensing cells (TCC) are modified epithelial cells, allocated in the taste buds considered the anatomical units for taste perception (*Figure 4*). Shen et al. (2005) defined TCC as primary sensory cells utilized by the nervous system to detect the presence of gustatory stimuli in the oral cavity. TCC from the same taste bud have been classified in four groups: type I are believed to be supporting glial-like cells controlling extracellular ion concentrations (sour taste) and are not involved in signal processing for taste sensation (Bigiani, 2001); type II represent the primary sensory receptive cells for taste stimuli transduced by guanine-coupled-nucleotide-binding protein coupled receptors or GPCR's (sweet, umami and bitter tastes) without synapses to afferent nerve fibers (Adler et al., 2000; Kaya et al., 2004); type III have afferent synapses and are responsible for the signal transduction from type I and II cells to the sensory neurons (Murray, 1986; Chen et al., 1996; Gilbertson et al., 2001; Caicedo et al., 2002; Palmer, 2007); and type IV are one basal-type or progenitor thought to be undifferentiated precursor cells for the renewal of the other three types (Farbman 1980; DeFazio et al. 2006; Romanov and Kolesnikov 2006).

The TCC type I and II perceive chemical signals through taste receptors (TRs) and produce changes in membrane potential. When taste buds were depolarized with KCl, or stimulated with bitter, sweet, umami or sour (acid) tastants, serotonin (5-HT) was released onto TRC (type III) and sensory neuron synapses (Kaya et al., 2004; Huang et al., 2005; Sugita, 2006). In mammals the transmitter of most taste cells making synapse with the gustatory nerve is 5-HT. This 5-HT release evoked by bitter, sweet and umami tastant stimulation requires Ca+2 influxes, then it seems to be triggered by intracellular Ca^{+2} releases (Huang et al., 2005; Sugita, 2006). The taste sensory neurons, in turn, transmit the taste information (intensity and quality) through the nucleus of the solitary tract of the medulla to the gustatory cortex in the brainstem and thalamus (Saper et al., 2002; Romanov and Kolesnikov, 2006; Sugita, 2006; Palmer 2007).

Figure 4 > Lingual epithelium showing the main structural and functional cells and other characteristic features associated with taste buds (drawing by Joaquim Roura, 2009).

1 Nerve fibers
2 Synapses
3 Type III sensory Cell
4 Basal cell
5 Supporting cell
6 Type II sensory cell
7 Microvilli
8 Outer taste pore
9 Epithelial cell
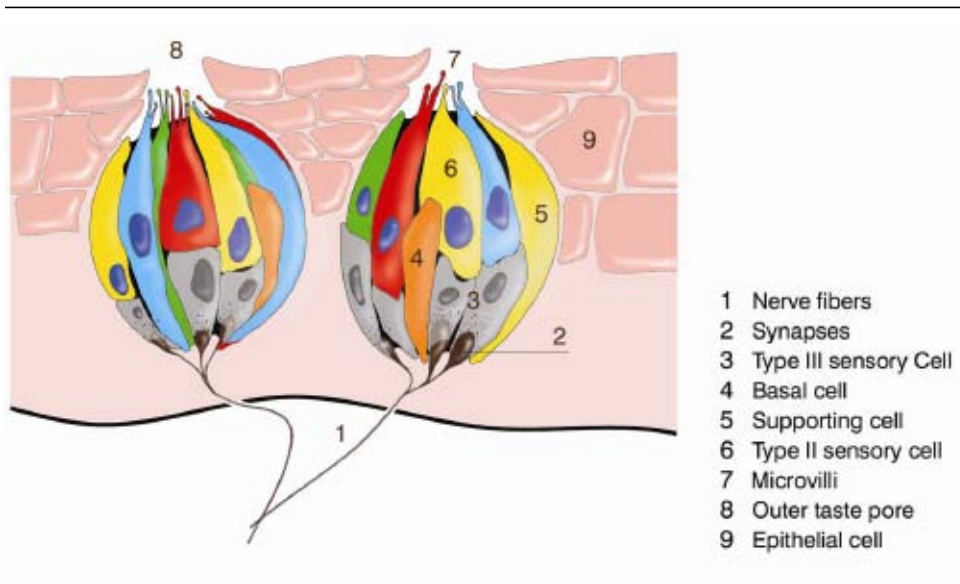
The TRs are transmembrane proteins expressed in the TCC microvilli that recognize specific soluble taste ligands present in the oral cavity (Gilbertson et al., 2000; Mombaerts, 2000; Matsunami and Amrein, 2003; Huang, 2006; Bachmanov and Beauchamp, 2007; Roura et al., 2008). Even though a taste bud may recognize all known different taste modalities (sweet, bitter, sour, salty and umami), any single TCC expresses only one family of TR (Dulac, 2000; Mombaerts, 2000; Huang et al., 2006) and the activation of a single type of TR is enough to encode a taste quality

(Chandrashekar et al. 2006). The TRs are responsible for detecting sweet, bitter, salty, sour and umami taste stimuli. Apart from these five "classic" tastes, a number of new candidate tastes have been recently proposed (*Table 6*) such as water, calcium and fat receptors with their respective TRs (Bachmanow and Beauchamp, 2007; Roura et al., 2008).

Table 6 > Taste receptor candidates and their known nutrient-related ligands in mammals (Bachmanow and Beauchamp, 2007; Matsumura et al., 2007; Roura et al., 2008).

TASTE PERCEPTION	TASTE RECEPTOR CANDIDATES[1]	NUTRIENT PROFILE	LIGANDS
Salty	ENaC[2] TRPV1[3]	Minerals and electrolytic balance	NaCl
Fatty	CD36, GPR120, GPR40[4]	Dietary lipids	Long-chain fatty acids
Starchy	Unknown	Starch and polysaccharides	Complex carbohydrates
Water	AQP5[5]	Hypo-osmotic fluids	Unknown
Unknown	CaR[6]	L-amino acids and calcium	L-aromatic amino acids, L-Glu and calcium.

1 - Taste receptor candidates from studies carried out in humans and rodents. / 2 - The selective epithelial amiloride-sensitive sodium. 3 - Variant of a vanilloid (capsaicin) receptor-1 proposed as amiloride-insensitive salt taste receptor. / 4 - The fatty acid transporter CD36 channel, GPR120 as receptor for unsaturated long–chain fatty acids in the intestine, GPR40 as receptor for long- and medium-chain fatty acids. / 5 - Aquaporin expressed applically in TRC. / 6 - Calcium-sensing receptor.

Apart from 5-HT, several known neurotransmitters like norephinephrine, acetylcholine and possibly GABA, some neuropeptides like neuropeptyde Y (NPY), cholecystokinin (CCK) and vasoactive intestinal polypeptide (VIP), glutamate (as a neurotransmitter and taste stimulus) and ATP have been suggested to play an important role in cell-to-cell communication in taste buds (Herness et al., 2002; Kaya et al., 2004; Shen et al., 2005; Zhao et al., 2005; Huang et al., 2006; Palmer, 2007). These transmitters are widely distributed in the central and peripheral nervous systems and they seem to play similar roles within the taste buds. For example, neuropeptides CCK and VIP exerts excitatory actions on single TCCs and NPY is just the opposite. It has been established that these neuropeptides are signaling agents that likely act in concert with transduction mechanisms to produce a final afferent signal that is relayed to the central nervous system (Zhao et al., 2005).

Main taste categories: salty, sour, bitter, sweet and umami

Each TCC type has a distinct intracellular taste receptor activated pathway. Salty and sour compounds activate TCCs through ion channels in the apical cell membrane while bitter, sweet and umami compounds act through more specific transmembrane TRs (Roura et al., 2008).

Salty is a taste mainly stimulated by ionic sodium, responsible for maintaining ion and water homeostasis. Salty is considered a pleasant taste except when it is too strong. It uses cation channels located mainly in the apical and basolateral cell membranes. The Na+ influx through the cation channels elicits membrane depolarization, leading to a consecutive cascade of events starting with the production of action potentials followed by neurotransmitter release that activates a voltage-gated K^+ channels of the afferent nerve fiber, Ca^{+2} inflow and subsequent synaptic exocytosis (Sugita, 2006). In rodents, a trimeric sodium channel receptor (ENaC) sensitive to the antagonist amiloride, also known as amiloride-sensitive receptor (Hettinger, 1990), has been identified. ENaC is associated with fibers of the CT nerve that respond specifically to Na salts. Approximately 65% of the taste cells in fungiform papillae exhibit functional amiloride-sensitive Na^+ channels (Lin et al., 1999). On the other hand, there is another type of ion channels that are amiloride-insensitive located in basolateral cell membrane. Amiloride-insensitive channels are associated to the other fibers from the CT and most of the GP taste nerve responses, and are mainly expressed in taste cells of the foliate and circumvallate papillae. These channels are derived from the vanilloid receptor-1 (VR-1) that is responsible for rejection-responses to K^+, $NH4^+$, and Ca^+ salts (Lyall et al., 2004, 2006; De Simone and Lyall, 2006). Candidate porcine VR-1 and ENaC receptors have been recently identified in *The Institute for Genomic Research pig EST database* (Roura and Tedó, 2009).

Sour taste responses are proportional to proton concentration. A broad range of cell types, receptors and mechanisms have been proposed to be responsible for sour taste. These include the activation of hyperpolarization-activated cyclic-nucleotide-gated channels (HCN), acid-sensing ion channels (ASICs), potassium channels (K2P), and H^+-gated calcium channels, as well as the involvement of Na^+/H^+ exchangers and acid inactivation of K^+ channels (Chandrashekar et al., 2006). These ion channels and also pH-dependent ion exchangers are located in the apical and basolateral cell membranes. The stimulation by acids elicits a membrane depolarization directly through the ion channels or by modulating the intracellular concentration of ions, such as Na^+, K^+ and Cl-. Membrane depolarization may be associated with the

production of action potentials through activation of voltage-gated channels, resulting in the release of neurotransmitter onto an afferent nerve fiber. The ASIC, the HCN, voltage-gated chloride channels (ClC) and polycystic kidney disease genes (PKD1L3 and PKD2L1) have been proposed as responsible for sourness perception (Lindemann, 2001; Huang et al., 2006; Ishimaru et al. ,2006; Sugita, 2006; Roura and Tedó, 2009).

Bitter, sweet and umami taste receptors are TR proteins that were discovered in the early 2000s (Mombaerts, 2000; Dulac, 2000). The TRs were identified by means of electrophysiology, molecular biology and genomics associated with behavioural studies (Sugita, 2006). These receptors belong to a superfamily named after guanine-coupled-nucleotide-binding protein coupled receptors (GPCRs). All GPCRs are transmembrane receptors characterized by 7 trans-membrane domains (*Figure 5*). They represent the largest superfamily in the human genome covering well over 1000 genes (accounts for approximately 2% of the genome) grouped into 3 main families (Chou, 2005): class A-"rhodopsin like", class B-"secretin like", and class C-"metabotrophic/glutamate/pheromone". Class C includes the taste receptors among many others (Lindemann et al., 2001; Chou, 2005; Niel et al., 2006). Taste GPCRs have been divided into two families: T1R and T2R (Mombaerts, 2000; Dulac, 2000; Matsunami and Amrein, 2003; Bachmanov and Beauchamp, 2007).

The T1R family contains three genes and belongs to the class C family of GPCRs, like the metabotropic glutamate and the pheromonal (also known as vomeronasal –VR-) receptors. The T1Rs receptor family (T1R1, T1R2 and T1R3) possess a large N terminal extracellular domain and generate at least two heteromeric receptors: the T1R1/T1R3 and the T1R2/T1R3, for umami and sweet tastes, respectively (Hoon et al., 1999; Nelson et al., 2001; Bachmanow and Beauchamp, 2007). The distinct ligand specificities of T1R1/T1R3 and T1R2/T1R3 receptors imply that T1R1 and T1R2 play more substantial roles in ligand binding of umami and sweet taste receptors than does T1R3 (Nelson et al., 2002; Bachmanov and Beauchamp, 2007). Moreover, T1R3 subunit might also be involved in the taste of calcium and magnesium (Tordoff et al., 2008). The T2R family is defined as the bitter taste receptors and the number of genes involved differs largely among animal species (Mombaerts 2000; Matsunami and Amrein 2003; Shi and Zhang 2006; Roura et al., 2008).

Bitter, sweet and umami tastes are initiated by the interaction of ligands with the G-protein coupled-receptors, the T2Rs and the T1R2/T1R3 and T1R1/T1R3 heteromers respectively (Chaudhari et al., 2000; Bachmanov and Beauchamp 2007; Hacker et al.,

2008; Roura et al., 2008). Apart from the T1R1/T1R3, other GPCRs for umami compounds have been identified in taste cells and suggested as umami taste receptors, the truncated type 1 (mGluR1) and 4 (mGluR4) metabotropic glutamate receptors (Toyono et al., 2003; Sugita, 2006; Yasuo et al., 2008, Chaudhari et al., 2009).

Both T1Rs and T2Rs are partially coexpressed with the α-subunit of the G protein gustducin (Gα) in TRs (Nelson et al., 2001, 2002; Scott, 2005; Sugita, 2006). The G protein gustducin is a taste-specific signalling molecule (Wong et al., 1996; Ruiz et al., 2003). Mice lacking Gα showed reduced responses to bitter, sweet and umami tastants, suggesting that Gα plays a role in taste transduction (Wong et al., 1996; Caicedo et al., 2003; Zhang et al., 2003; He et al., 2004). The α-Transducin (Tα) is another taste-specific signalling molecule expressed in TRs at much lower level, but it is only involved in response to umami compounds (He et al., 2004).

Figure 5 > The guanine-coupled-nucleotide-binding protein taste receptors: umami, sweet and bitter (from Chandrashekar et al., 2006).
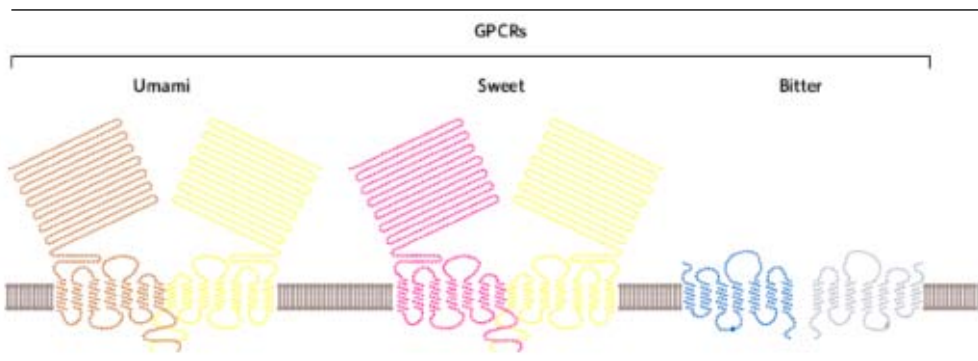
The G protein gustducin have other two subunits more, β (Gβ) and γ (Gγ). The Gβ3 and Gγ13 are also expressed in TRs and are responsible of the phospholipase C (PLCβ2) activation (Rossler et al., 1998; Huang et al., 1999).The PLCβ2 and the transient receptor potential M5 ion channel (TRPM5) are two signal transduction molecules expressed in sweet, umami and bitter TRs (Miyoshi et al., 2001; Prawitt et al., 2003; Zhang et al., 2003; Sugita, 2006).

There is a common transduction pathway for sweet, umami and bitter taste that starts with a ligand binding to the TRs. The cascade continues through Gα (and/or Tα for the umami taste) to activate the PLCβ2-dependent pathway, which catalyzes the formation of inositol triphosphate (IP3) and Dyacilglicerol (DAG), leading to a

release of calcium from intracellular stores. Alternatively, it may be activated a cAMP-dependent pathway (Gilbertson et al., 2000; Margolskee, 2002). This intracellular calcium release activates the TRPM5 and results in the entry of monovalent cations (Na^+), membrane depolarization and generation of action potential of taste cells (Adler et al., 2000; Dulac, 2000; Mombaerts, 2000; Firestein 2001; Montmayeur et al., 2001; Li et al., 2002; Nelson et al., 2002; Liu and Liman, 2003; Matsunami and Amrein, 2003; Prawitt et al., 2003; Sugita, 2006; Yasuo et al., 2008) (*Figure 6*). Only some compounds, including known bitter and sweet tastants, are able to penetrate the cell without requiring any specific GPCR (Naim et al., 2002; Sawano et al.2005).

Figure 6 > The common transduction pathway for sweet, umami and bitter taste (from Sugita, 2006).
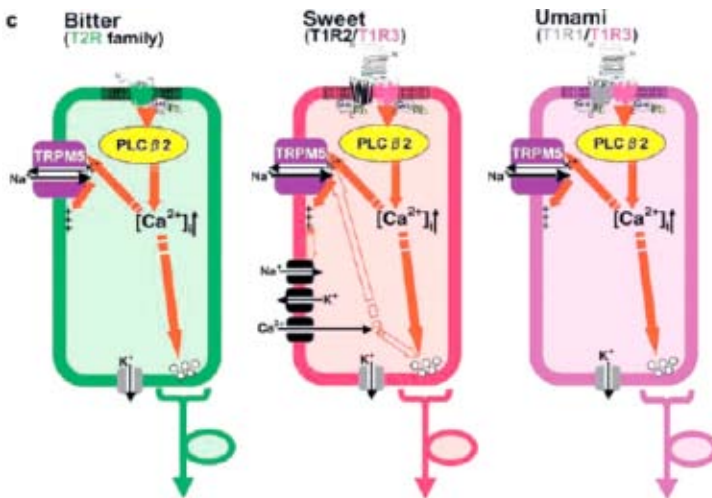
There is only one known receptor for sweetness, the heterodimer T1R2/T1R3 (Matsunami et al., 2000; Margolskee, 2002; Li et al., 2002). The sweet TR heterodimer recognizes a large collection of diverse chemical structures like sugars, some D-amino acids, artificial sweeteners and some proteins that have been described as sweet compounds in human, rat and mouse (Li et al., 2002; Nelson et al. 2002; Bachmanov and Beauchamp, 2007). The T1R2/T1R3 double knockout mice do not display behavioural and nerve responses to sweet stimuli, supporting the functionality of T1R2/T1R3 as the sweet receptor (Li et al., 2002). Nevertheless, other studies in single and double knockout mice of T1R2 and/or T1R3 suggested that T1R2 and T1R3 may act as a monomer under high concentrations of natural sugars (Zhao et al., 2003) or other sweet taste receptors may exist (Delay et al., 2006).

The umami taste, also known as the monosodium glutamate (MSG) taste (Ikeda, 1909), is perceived by the heterodimer T1R1/T1R3 and other metabotropic glutamate receptors, mGlu1 and mGlu4 (Nelson et al., 2002; Backmanov and Beauchamp, 2007; Roura et al., 2008; Yasuo et al., 2008; Chaudhari et al., 2009). Mouse models reported that umami responses were abolished or reduced in T1R3 and T1R1 knockout mice (Damak et al., 2003; Zhao et al., 2003). The main substance eliciting umami taste in humans is L-glutamate, an amino acid widely present in food. In general, the umami taste is mainly related to protein, peptides and L-amino acids, and it is enhanced by 5'-ribonucleotide monophosphates such as inosine (IMP) and guanosine-5'-monophosphate (GMP) (Ninomiya and Funakoshi, 1989; Yamaguchi, 1991; Ninomiya, 2002; Backmanov and Beauchamp, 2007; Roura et al., 2008b).

There is a genetic variation in taste that influences food selection (García-Bailo et al., 2008), natural allelic variation and common polymorphisms of T1R's may be responsible of variation in taste responses that may explain sensory differences, within and between populations (Max et al., 2001; Reed et al., 2004; Kim et al., 2006; Reed et al., 2006; García-Balio et al., 2008). Several polymorphisms in T1R3, T1R1 and T1R2 have been described in human (Kim et al., 2006; Raliou et al., 2009) and mouse (Red et al., 2004; Inoue et al., 2007) that may explain differences in dietary preferences.

Although the porcine genome is not still available, Roura et al. (2008a) have recently reported the sequence of the porcine umami T1r1 and T1r3 genes. The porcine umami TRs show higher nucleotide and amino acid sequence homologies with carnivore mammals (e.g. the cat) than with human and mice (Humphrey et al, 2009). Therefore, the extrapolation of umami taste perception from humans and mice may not be a good model for an understanding of the pig taste (Humphrey et al., 2009).

The T1R family expression in non-taste tissues

In general, taste perception mechanisms are associated to mouth taste buds through TCC. Thus, tongue's TCC are believed to be the main sites of the expression of TRs genes. The T*as*1r3 gene is expressed in all type of taste buds in several species, including pigs (Kiuchi et al., 2006; Bachmanov and Beauchamp, 2007; Roura et al., 2008b). The T*as*1r1 gene expression in rats and mice is predominantly present in the fungiform papillae and palate taste buds, at a lower level in foliate and rarely present in circumvallate papillae, just the opposite to the distribution of T*as*1r2 gene expression (Hoon et al., 1999; Kitagawa et al., 2001; Montmayeur et al., 2001, Nelson et al., 2001). Moreover, T1R genes are coexpressed in TCC and seem to function as heterodimers, T1R1/T1R3 and T1R2/T1R3, for umami and sweet taste, respectively (Bachmanov and Beauchamp, 2007).

According to Sbarbati and Osculati (2005), taste buds are only the most visible portion of a network of a chemosensory system spread through most body tissues in the form of solitary chemosensory cells (SCCs) or chemosensory clusters. That chemosensory system would have been evolved from a very ancient highly conserved system involved in defensive and nutritive mechanisms (Roura et al., 2008b; Sbarbati et al., 2009).

Recent findings have demonstrated the expression of TR's in several non-taste tissues, mainly from the digestive system, referred to as ectopic TR's expression (Dyer et al., 2005; Sbarbati and Osculati, 2005; Kiuchi et al., 2006; Bachmanov and Beauchamp, 2007; Rozengurt and Sternini, 2007; Sternini et al., 2008, Roura et al., 2008). A mechanism involving TR coupled to G proteins and increases in intracellular Ca+2 induction, has been postulated that would result in a release of peptides that regulate a wide variety of GI functions, and also control food intake through the gut-brain axis (Sternini et al., 2008). (*Figure 7*).

Figure 7 > Postulated mechanisms involving T1R and T2R taste receptors on enteroendrocine cells (from Sternini et al., 2008).
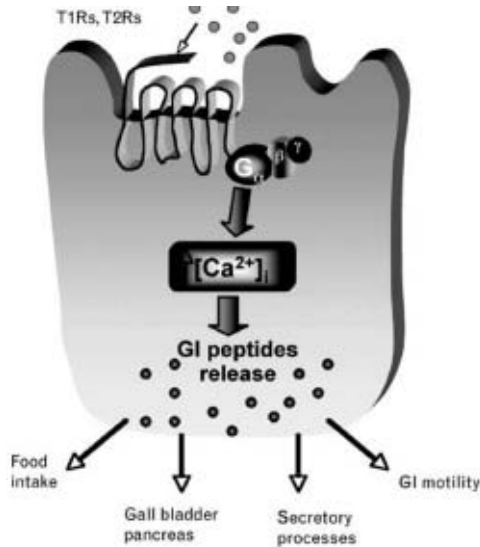
The SCCs are present in many different tissues forming a diffuse chemosensory system (DCS) that shares common signaling mechanisms with TCC. The intestinal brush cells or SCC have a structure similar to lingual TCC and strongly express α-gustducin (Mace et al., 2007). Bezençon et al. (2007) determined in humans and mice that a similar taste chemosensory system exists in the GI tract, showing that T1R1, T1R2, T1R3, G_{α},gust, PLC β-2 and TRPM5 were expressed in the stomach, small intestine and colon, suggesting that these cells may be involved in sensing nutrients (Becençon at al., 2007; Mace et al., 2007). The expression of these genes in the GI tract may involve them in the control of food intake through the activation of gut–brain neural pathways. Sternini et al. (2008) suggested that these findings provide a new dimension to unravel the regulatory circuits initiated by luminal contents of the GI tract.

In mice, the T*as*1r2 gene encodes the receptor protein for sweet taste perception but in the GI mucosa the gene is also transcribed and has been associated with active glucose transport (Dyer et al. 2005, 2007). Natural and artificial sweeteners stimulated T1R2 and T1R3 in the brush-border intestinal cells of mice and rats. The T1R2/T1R3 activation resulted in increased expression of the glucose transporter type 2 (GLUT2) (Mace et al., 2007) and the sodium-dependent glucose transporter isoform 1 (SGLT1) (Dyer et al., 2007; Margolskee et al., 2007). It has been suggested that this intestinal cells may participate in sugar sensing by a TR mediated mechanism

analogous to taste buds (Mace et al., 2007, Margolskee et al., 2007). Other studies also showed T1R3 expression in other non-taste organs, such as testis, brain, thymus, enteroendocrine cells, kidney, lymphocytes, liver and pancreas (Kitagawa et al., 2001; Max et al., 2001; Taniguchi, 2004; Kiuchi et al., 2006; Sbarbati et al., 2009).

In pigs, the T1R3 (pT1R3) has been shown in several tissues with a similar expression profile than mouse, except for kidney (*Figure 8*). Kiuchi et al. (2006) showed that tongue expressed the pT1R3 at a much higher rate than other tissues. Stomach also expressed this gene significantly more than intestine and liver. In general, tissues reported that had the highest level of expression of pT1R3 were tongue (in circumvallate and fungifrom papillae, mucosal epithelial cells, lymphocytes in submucosal tissues of the lingual tonsil and follicular B lymphocytes), testis (spermatogenic cells), and kidney. The presence of this gene in non-taste tissues of pigs suggests that the taste receptors may be involved in the chemosensory function of these organs participating in several behavioural, digestive and metabolic processes.

Cell reporter systems to study the umami ligand specificity

The G-protein-coupled receptors (GPCRs) constitute the largest family of trans-membrane proteins. They respond to a wide variety of ligand structures and mediate most transmembrane signal transduction in response to hormones and neurotransmitters but also in response to the senses of sight, smell, taste and pheromones. Individual GPCRs can signal through several G-protein subtypes and through G-protein-independent pathways, often in a ligand-specific manner (Minic et al., 2005; Kobilka and Deupi, 2007).

Figure 8 > Measurements of T1R3 gene expression in porcine tissues by real-time PCR. The pT*as*1r3 tissue expression was calculated relative to liver (from Kiuchi et al., 2006).
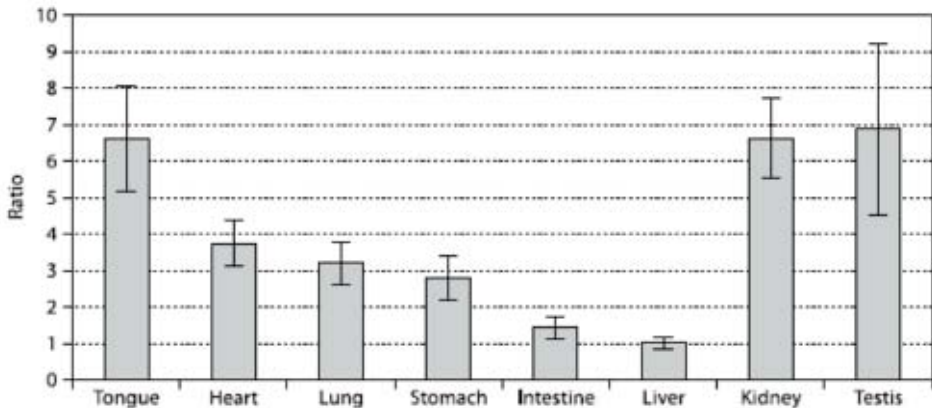
Heterotrimeric G proteins are classified into four families based on the amino acid sequence homologies of their Gα subunit: Gs, Gi, Gq, and G12. The Gs family comprises Gs and the 'olfactory' Golf. Common to Gs family members is their capability to activate adenylyl cyclases, causing an increase in intracellular 3',5'-cyclic adenosine monophosphate (cAMP). The Gi family is most diverse, and consists of Gαi1, Gαi2, Gαi3, Gαo, Gαt-r, Gαt-c, Gαg, and Gαz. 'Rod transducin'Gαt-r, 'cone transducin' Gαt-c, and 'gusducin' Gαg are involved in the transduction of visual and taste signals. The sensory Gi protein Gαg activates PLCβ and induces increases in intracellular Ca2+, whereas Gαt-r and Gαt-c activate the cGMP-dependent phosphodiesterases (PDE) 8, causing a decrease in intracellular cGMP and hyperpolarization of photocells via cGMP-gated ion channels. The Gq family includes Gαq, Gα11, Gα14, and Gα15/16. All members are coupled to activation of the four Phospholipase C isoforms (PLCβ1–4), and hence cause activation of protein kinase C (PKC) and intracellular Ca^{2+} mobilization. Gαq and Gα11 are ubiquitously expressed, whereas the expression of Gα14 is more restricted. Gα15 and Gα16 are solely found in hematopoietic cells, and resemble the mouse and human orthologs of the same gene. The more recently described G12 family consists of the ubiquitously expressed Gα12 and Gα13 proteins. Both activate the small GTPase Rho. Some GPCRs couple solely to one type of G protein such as Gi or Gs, whereas most receptors couple to a broader range of G proteins such as Gi, Gq, and G12. Exceptions to typical coupling profiles exist, and the strength of coupling to one versus another G protein can differ (Birnbaumer and Birnbaumer, 1995; Offermanns, 2003; Sujatha et al., 2003; Siehler, 2008) (*Figure 9*).

Figure 9 > Mammalian G protein families (Gs, Gi, Gq, G12) and typical G protein coupling profiles of GPCRs. Each family activates specific signal transduction molecules shown into squares[1]. (Adapted from Siehler, 2008).
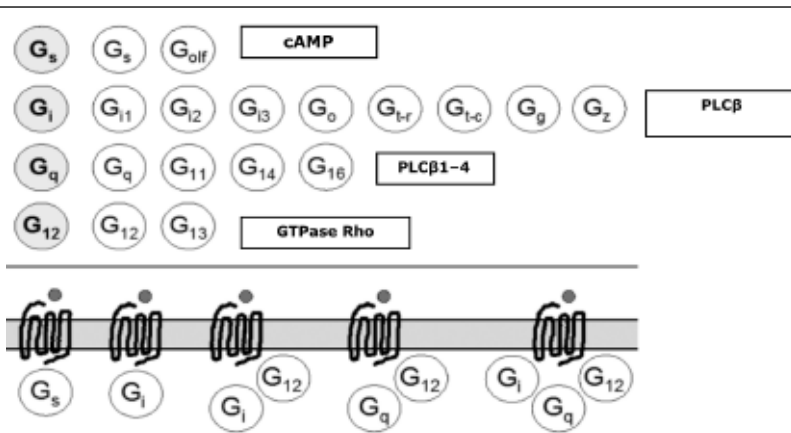
1- intracellular cyclic adenosine monophosphate (cAMP), Phospolipase C β (PLCβ 1-4), intracellular cyclic guanosine monophosphate -dependent phosphodiesterases 8 (cGMP-PDE8) AND Guanosin-triphosphatase Rho (GTPase Rho).

Detection methods of ligand-GPCRs binding interactions have moved a long way from membrane-based radioligand binding assay towards cell-based functional assays. Several eukaryotic expression systems have been used for screening of agonists and antagonists of different GPCRs (-Yeast- Minic et al., 2005; -Chinese ovary hamster-K1 and Human Embryonic Kidney 293 cells- Zhu et al., 2008). Since GPCRs are major targets for potential therapeutic agents, development of robust, reliable and cost effective screening methods for these receptors have been used for the assessment of GPCR modulators (Shiehler, 2008; Zhu et al., 2008; Kurko et al., 2009; Rodrigues and Mcloughlin, 2009). These methods, known as GPCRs' high throughput screening processes (HTP), have been designed to rapidly identify active compounds that can modulate a particular biomolecular pathway, especially focused on the drug discovery (Lundstrom, 2006; Nanda et al., 2009).

These systems of screening are measuring GPCRs' second messenger signals, such as an increase in intracellular calcium level (Hanson, 2006; Nanda et al., 2009), changes in intracellular cyclic adenosine monophosphate (cAMP) or inositol triphosphate (IP3) (Eglen, 2005; Selkirk et al., 2006; Rodrigues and Mcloughlin, 2009), which normally include a reporter gene technology. Gene reporter assays are based on identification of changes in intracellular concentration of second messenger signals through changes in the level of the gene reporter expression (Eglen et al., 2003; Kunapali et al., 2003; Parmesh et al., 2004; Chepurny and Holz, 2007). Gene reporter expressions are regulated by specific transcriptional factors such as cAMP response element binding protein (-CREB-, Selkirk et al., 2006; Chepurny and Holz, 2007), serum response element binding factor (-SRF-, Parmesh et al., 2004) and nuclear factor of activated T cells (-NFAT-, Hanson, 2006). On the other hand, some of the reporter genes used in GPCRs' assays are β- galactosidase, β-lactamase and Luciferase (Hill et al., 2001; Yan et al., 2002; Eglen and Singh, 2003; Kunapali et al., 2003; Parmesh et al., 2004; Hanson, 2006; Chepurny and Holz, 2007; Bercher et al., 2008). Finally, several fluorescence-based ligand binding assays have been developed and used in HTS of GPCR targets. The most common is to analyze the level of reporter gene expression through resonance energy transfer techniques, which allow evaluation and characterization of the receptor-ligand binding interactions. Many resonance energy transfer techniques utilise a fluorescent protein as the energy acceptor. In bioluminescence resonance energy transfer (BRET) the energy donor is a luciferase that generates light by oxidation of a substrate, whereas in fluorescence resonance energy transfer (FRET), if the acceptor is an fluorescent protein, a second fluorescent protein with distinct spectral characteristics acts as the energy donor (Milligan, 2004; Hu et al., 2008).

The T1R taste receptor family comprises three GPCRs that use diverse signaling pathways, the α-Gustducin mediated PLC β2-dependent pathway, or the cAMP-dependent pathway (Li et al., 2002; Margolskee et al., 2002; Nelson et al., 2001, 2002). Taste cells use two distinct signaling pathways that result in the release of the neurotransmitter ATP: by calcium influx through voltage-gated calcium channels (VGCCs), or by G protein–coupled receptor (GPCR)-dependent second messenger calcium release from intracellular stores (DeFazio et al., 2006; Huang et al., 2007; Hacker and Medler, 2008). Increases in cytosolic calcium are critical for normal responses in both pathways (Hacker and Medler, 2008).

Nelson et al. (2002) used a cell expression screening strategy to identify taste receptors involved in amino-acid detection based on the heterologous expression of candidate receptors in a cell culture and calcium imaging analysis by fluorescence. Candidate receptors, promiscuous G proteins and heteromeric mouse T1R1[+3] or T1R3 and T1R1 alones, were co-expressed in human embryonic kidney (HEK) cells and assayed for stimulus (D- and L- amino acids at 50mM with or without IMP at 2.5mM, among others) that evoked changes in intracellular calcium. In this system, receptor activation leads to activation of PLC β2 and release of calcium from internal stores, which can be monitored at the single-cell level using calcium-indicator dyes. The results obtained from this cell reporter system showed that responses were dependent on the combined presence of T1R1 and T1R3. This combination was very selective for L-amino acids, its response was enhanced by IMP and no responses were observed with D-amino acids, even combined with IMP. They concluded that T1R1 and T1R3 combine to function as a broadly tuned L-amino acid sensor.

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Objectives

Objectives >>>>>>>>

The present work has the following objectives:

Objective 1

To study short-term dose-response preferences for L-amino acids and Glycine in non-isolated weaned piglets with "ad libitum" access to feed and water.

Objective 2

To study the dose-dependent stimulation of the porcine umami taste receptor (pT1r1/pT1r3) by L-amino acids and glycine.

Objective 3

To compare the data on amino acid preference obtained from pig in vivo trials with those recorded by the porcine umami cell system.

Objective 4

To study the expression of the pig umami taste receptor in several taste and non-taste tissues of the gastrointestinal tract as a function of age, sex and the dietary crude protein content.

The hypotheses of our work were (1) that pigs have the capacity to taste amino acids known as umami in other mammalian species, (2) that such taste is perceived through the heterodimer T1r1/T1r3 receptor system, (3) that the pT1r1/pT1r3 in pigs functions as an amino acid sensor in the tongue and the gastrointestinal tract, and (4) that their expression is affected by sex, age and dietary crude protein content.

Chapter 1

Individual and pair double-choice test models to evaluate amino acid preference and appetence in weaned pigs under fed-status

Chapter 1 >>>>>>>>

Abstract

Multiple choice models to assess taste perception have been used to evaluate the appetence for solutions of nutrients and other tastants in many animal species, being mainly concentrated on determination of thresholds. Pig models include training sessions of 3-5 days before conducting experimental tests where animals were permanently housed individually to get them adapted to social isolation and/or maintained fasted to ensure animal interest for the test. The aim of this work was to evaluate pig preferences, consumption and appetence under a fed-state for Glycine and several L-AA at different concentrations. In order to achieve the main objective, the development of a reliable *"in vivo"* model for double choice testing in piglets avoiding the isolation time and fasted state of pigs was also pursued. Six experiments were conducted, two of them using an individual double-choice test (DOCH) model (eighty-two pigs) where animals were only isolated from penmates during training and test sessions. In this model, training sessions lasted 4 days and 500mM amino acid solutions were tested. The other four experiments used a pair DOCH model (ninety-six pigs) always maintaining animals in pairs (1 male and 1 female). In this model, training sessions only lasted 2 days and four different concentrations (0.5, 5, 50 and 500mM) of amino acid solutions were tested. In both models, pigs were under an operant conditioning scheme of two 10-minute training sessions (9 am and 12 pm) every day where the DOCH was between 250g of either tap water (control solution) or a 500mM sucrose solution (test solution) that were switched every day. On the last training day, animals showing no attempts to escape, a latency time of less than 30 seconds, no vocalizations and 50g/pig of minimum consumption were selected for the following 2-minute DOCH tests. The 500mM sucrose and tap water test solutions were both used as positive and negative controls, respectively, in test sessions. Amino acid palatability was studied using preference values (in % -P-), consumption rate of test solution (in g/s –CRT-) and rate of appetence (in g/s –RAp-) defined as: RAp=(0.001+P)*(CRT). In

the individual model, only forty-one pigs were selected following the behavioural and consumption criteria, whereas when housed in pairs none of them were excluded. Differences between water and the positive control (P<0.01) were showed in the pair model, proving that only pigs under this DOCH procedure were able to discriminate between them. Results of tested amino acids at 500mM in individual and pair DOCH models showed that monosodium glutamate (MSG) and glutamic acid (L-Glu) were the most preferred and had the highest P and RAp (P<0.01), similar to sucrose (positive control). The L-tryptophan (L-Trp) was the least preferred and appetent amino acid (P<0.01). Other data from the pair model showed a clear dose-response in P, CRT and RAp for L-Thr and L-Trp. Their preference, consumption and appetence for them increased at low and decreased at high concentrations (P<0.001). A negative correlation was observed between P, CRT and RAp and the number of animals consuming less than 50g during test sessions (r= -0.375; r=-0.489 and r=-0.387, respectively; P<0.001). Animals were not motivated to make the choice test with less preferred amino acid concentrations. In conclusion, individual 2-minute double-choice test model avoiding an adaptation period to isolation might be adequate for studying preference and appetence by amino acids if pigs are previously selected based on their behaviour and consumption, and if time of training is enlarged. The pair model may be more appropriate to prevent the social isolation effect on pig learning capacity for DOCH testing. Weaned pigs under a fed-status were able to discriminate solutions of amino acids except when those were offered at 0.5mM. In general, piglets showed significant preference and appetence for non-essential amino acids (L-Glu and MSG). However, this taste response changed with essential amino acids, resulting in significant aversions at high concentrations (L-Trp, L-Thr).

Introduction

One of the most important priorities in piglet nutrition is to prevent weaning anorexia or low levels of feed and water intake just after weaning. Optimal nutrition is more than the supply of adequate amounts of protein, energy, vitamins and minerals. Ingredient selection based not only on proper nutritional composition but also on palatability cues may be very important to obtain appropriate feed intake of a highly digestible well balanced diet, particularly in young animals such as the weaned piglet. The sensorial perception enables an animal to recognize sources of nutrients indicating the nutritional value of potential foods (Goff and Klee, 2006). It is also interesting to know the ability of the animal to discriminate between concentrations of different tastants, which might be of good nutritional value but become toxic depending on their intake levels (Laska et al., 2008). The impact of changes in feed ingredients on dietary preferences or aversions in pigs have been studied by choice-feeding trials (Solà-Oriol, 2008). Double-choice studies in pigs were also used to compare different levels of a particular nutrient in a diet and diet complexity (Kyriazakis, 1995; Morgan et al., 2003; Ettle and Roth, 2004; Ettle and Roth, 2005; Roth et al., 2006; Solà-Oriol, 2008).

Other studies in pigs showed preferences for human sweet and umami tastants using an individual *"in vivo"* double-choice test where animals were adapted to social isolation and fasted before being challenged (Kennedy et al, 1972; Glaser et al., 2000; Tinti et al., 2000; Nofre et al., 2002). The umami taste is a pleasant taste related to protein born nutrients such as amino acids and oligopeptides that indicates the presence of protein (Ninomiya, 1998; Bellisle, 1999). Furthermore, electrophysiological recordings of the *chorda tympani* (CT) and glossopharyngeal (GP) nerves, through which taste information is conveyed from taste buds, have been used in pigs to evaluate the intensity of gustatory responses (Hellekant and Danilova, 1999). Danilova et al. (1999) found that umami was a very strong taste stimulus for the pig and was predicted to provide a positive hedonic response, suggesting that umami tastants, such as monosodium glutamate (MSG) and other L-amino acids (L-AA), may promote feed intake. This method often corroborates the results from pig double-choice tests (Glaser et al., 2000; Tinti et al., 2000), although these recordings require invasive procedures, may involve cell damage, often yield relatively small sample sizes (Caicedo et al., 2002), and do not allow to know which taste receptor and type is being stimulated.

The aim of this work was to evaluate pig preferences, consumption and appetence under a fed-state for Glycine and several L-AA at different concentrations. In order to achieve the main objective, the development of a reliable *"in vivo"* model for double choice testing in piglets avoiding the isolation time and fasted state of animals was also pursued.

Materials and methods

Six experiments were conducted at Lucta S.A.- University of Girona, Swine Experimental Unit (SEU) "El Castell" (Sant Aniol de Finestres, Girona, Spain). Two of the trials followed an individual double-choice test model where pigs were only isolated from penmates in test pens during training and test sessions. The other four experiments were performed following a pair double-choice test model where animals were always maintained in pairs. Individual and pair double-choice tests (DOCH) models were approved by the Autonomous University of Barcelona Animal Care and Use Committee.

Animals

One hundred seventy-eight weaned piglets were used in DOCH tests. In the individual model, eighty-two piglets (41 males and 41 females, Landrace x Largewhite, 10.55±1.48Kg BW, 36±3 day-old) were trained. In the pair model, ninety-six piglets (Landrace x Largewhite, 10.73±1.20Kg BW, 38±3 day-old) were distributed in groups of two penmate pigs, 1 male and 1 female, in each pen of test.

Facilities

Animals from the individual model were penned in groups of four (0.56 m²/pig) two days prior to the training sessions. Animals from the pair model were kept in the same test pens throughout the experiment (1.22m²/pig). Each pen was equipped with two nipple-drinkers and one feeder with four heads that allowed eating at the same time. The experimental room was equipped with video-cameras (Circontrol®, Spain). Environmental conditions were automatically controlled (Skov®, Denmark) maintaining temperature at 28.5ºC and 25ºC with 60-65% of humidity at the start and at the end of the experiments (approximately 2 weeks), respectively. Animals had 2 days to get habituated to the facilities and the personnel before starting the DOCH protocol. In the individual model, pigs were moved from group pens to test pens into the same experimental room (twelve identical pens per room) and maintained isolated from penmates during training and test sessions, except for the possibility of visual and auditory contact with conspecifics. In the pair model, two identical experimental rooms were used to conduct each experiment. Piglets had ad libitum access to feed and tap water in both models.

Feeding

Animals were fed ad libitum with a commercial medicated pelleted starter diet. Feed was changed every day to maintain it as fresh as possible. Drinkers and feeders were checked daily to ensure continuous supply of water and feed to the animals. The composition of the experimental diet had 2.521 Kcal of NE*Kg $^{-1}$ of DM, 19.10% Crude Protein, 1.30% dLys, 0.50%dMet, 0.28% dTrp and 0.80% dThr (*Table 1*).

Experimental models

Training period

Double-choice tests were adapted from Glaser et al. (2000). The individual and pair DOCH tests consisted of a 10-minute training period of 4 and 2 days, respectively, using water (control) and a 500mM sucrose solution (test) offered in unfamiliar stainless steel containers. The two containers were offered daily side by side at 9am and at 12pm, switching their position to prevent a position effect and to ensure that piglets learnt to make a choice.

Table 1 > Composition of the diet (as fed basis)

INGREDIENTS	G*KG⁻¹	ESTIMATED NUTRIENT COMPOSITION	
Wheat	350.00	Crude Protein (g*Kg⁻¹)	191.40
Corn	200.00	Crude Fiber (g*Kg⁻¹)	34.40
Barley	170.33	Fat (g*Kg⁻¹)	61.50
Soy bean meal 47.5%	150.39	Ash (g*Kg⁻¹)	43.90
Soy oil	39.20	Energy (Kcal NE pigs*Kg⁻¹)	2521.00
Fishmeal 70%	30.00	Calcium (g*Kg⁻¹)	7.00
Premix – L-Tryptophan[1]	20.00	Inorganic P (g*Kg⁻¹)	7.59
Monocalcium Phosphate	7.60	Chloride (g*Kg⁻¹)	3.38
L-Lysine 50%	7.30	Sodium (g*Kg⁻¹)	2.00
Calcium carbonate	6.20	Methionine (g*Kg⁻¹)	4.75
Salt	4.10	Met+Cys (g*Kg⁻¹)	8.02
Acidifier	2.00	Lysine (g*Kg⁻¹)	13.20
L-Threonine 85%	2.00	Tryptophan (g*Kg⁻¹)	2.88
D,L-Methionine 83%	1.80	Threonine (g*Kg⁻¹)	8.85
Coline Chloridrate 75%	1.70		
Water	0.90		
Phytases	0.10		

1 - contains vitamins and minerals, L-Trp, 300 mg*Kg⁻¹ Amoxicilin Trihidrate and 80 mg*Kg⁻¹ Colistin Sulphate. Vitamins and minerals per Kg of diet: Vitamin A, 10000 IU,; Vitamin D3, 2000 IU; Vitamin E 15mg; Vitamin B1, 2 mg; Vitamin B2, 7 mg; Vitamin B12, 0.030 mg; Vitamin K3, 2 mg; D-calcium Pantothenate, 30 mg; Nicotinic acid, 50 mg; Biotin, 0.1 mg; Folic acid, 1.2 mg; Fe, 70 mg as iron carbonate; Cu, 165 mg as copper sulphate pentahydrate; Co, 0.14 mg as cobalt carbonate; Zn, 125 mg as zinc oxide; Mn, 43 mg as a manganese sulphate; I, 0.74 mg as potassium iodate; Se, 0.30 mg as sodium selenite; Etoxiquin, 100 mg.

The last day of training, behavioural parameters were recorded and consumed solutions (test and control) were measured to select the performing piglets. Pig behaviour (focal sampling) was recorded by direct observation of always the same observer and video-cameras (Circontrol®, Spain), and consumption was measured weighting containers in a scale (Kern model, Mettler Toledo®, Spain) before and after each 10-minute period. The

behavioural parameters were attempts to escape (AS), latency time (TL) and vocalizations (V) (*Table 2*). It was developed a score to assess the level of adaptation of pigs to the experimental conditions and to select them for the following testing sessions. This score considered values obtained from behavioural parameters and total consumption during training sessions. The final score was the addition of every value from behavioural and consumption parameters (*Table 2*). Pigs were excluded for testing when their score was higher than 5. In both models, piglets had 2 days to be adapted to researchers, experimental rooms, pen facilities, environmental conditions, feed and water supplies before training session start.

Table 2 > Behavioural and consumption criteria to select the piglets best adapted to the test conditions.

PARAMETERS	DEFINITION	EVALUATION	SCORE*
Latency time (TL)	Time it took piglets to touch both containers	Adaptation to novelty	< 30s=0 >30s=1
Vocalizations (V)	Number of vocalizations that piglets emitted	Adaptation to social isolation	No=0 Yes=1
Attempts to escape (AS)	Number of jumps identified as the animal trying to escape from confinement	Adaptation to social isolation	No=0 Yes=1
Total consumption (TC)	Total intake (g) adding both control and test solutions	Adaptation to novelty and social isolation	>200g=1 100-200g=2 50-100g=3 <50g=4

* The final score results of the addition of every value from every evaluated parameter.

Test period (testing sessions)

Selected piglets performed daily two 2-minute double-choice tests, at 9 a.m. and 12 p.m., during 5 consecutive days, normally a non-essential amino acid (Gly, L-Ala, L-Gln, L-Glu and MSG) and an essential amino acid (D,L-Met, L-Lys, L-Thr and L-Trp). Test solutions were distributed among animals or pens randomly, switching containers every time in a random left-right position. Consumption of test and control solutions was measured weighting containers before and after each 2-minute period in a scale (Kern model, Mettler Toledo®, Spain). Test solutions were prepared daily. Negative (tap water against tap water) and positive (500mM sucrose solution against tap water) treatments were always carried out at the beginning of the test sessions. In weekends, in order to maintain the animals trained, one positive 2-minute DOCH test with sucrose was offered daily between 9a.m. and 12p.m. roughly around 250g was the quantity offered in each container.

Tested amino acids were L- Lysine (L-Lys) (Quimidroga, Spain), D,L-Methionine (D,L-Met) (Andrés Pintaluba, S.A., Spain), L-Threonine (L-Thr) (Andrés Pintaluba, S.A., Spain), L-Tryptophan (L-Trp) (Quimidroga, Spain), L-Glutamic acid (L-Glu) (Materias Químicas, S.A., Spain), L-Glutamine (L-Gln) (Materias Químicas, S.A., Spain), Monosodium L-glutamate (MSG) (Materias Químicas, S.A., Spain), L-Alanine (L-Ala) (Materias Químicas, S.A., Spain) and Glycine (Gly) (Altaquímica, S.A., Spain), all of them were assigned to animals and pens randomly. The amino acids where tested at a single dose of 500mM in the individual model (except L-Ala and L-Thr), and at multiple doses of 5, 50 and 500mM in the pair model. The 0.5mM concentration was also tested in the pair model in some amino acids: Gly, L-Ala, L-Gln, L-Lys, L-Trp and MSG. The pH was measured in all the amino acid and sucrose solutions and water (model 713THpHmeter, METROHM®, Switzerland).

Calculations of preference, consumption rate of test solution and appetence

Both models, the quantity of the disappeared solution from each container (control and test) during the 2-minute tests were used to calculate the preference (P), consumption rate of test solution (CRT) and rate of appetence (RAp) as follows:

P = g of test solution / (g of test solution + g of control solution)
CRT = g of test solution /120s (g/s)
RAp = (0.001+P)*CRT (g/s)

Statistical analysis

Comparison between models (individual vs pair)

Behavioural data, 10-minute total consumption (test+control) and score of both models were analysed to compare the level of adaptation of pigs to the experimental conditions and to show the differences between each model. Behavioural parameters, total consumption and the score of both models were compared separately. Differences between models in terms of TL, V, AS and total consumption (*Table 2*) were analysed with a chi-square test and the Score comparisons were performed by the Wilcoxon test. Significance p-values were considered at p<0.05. All the statistical analyses were carried out with the statistical package SAS® (version 9.1., Software SAS Institute Inc., Cary NC, 1991-2001).

DOCH tests

The experimental unit in the individual model was the animal while in the pair model was the pair of animals. To evaluate amino acid preferences in piglets, P, CRT and RAp were used as dependent variables of our study. Data from individual and pair models were analysed separately. The P, CRT and RAp values were compared using the MIXED procedure with animal (individual model) or pen (pair model), and trial treated as random effects. Least square means (LSMEANS) were separated into significant main effects by the PDIFF option and applying the Tukey correction (P<0.01 due to multiple comparisons). Data from animals or pens that did not reach a minimum total consumption of 50g were not included in the statistical analysis and therefore were treated as missing values. Data and residuals from P, CRT and RAp were transformed using the arcsinus or Log10 transformations to obtain normality. Pearson's correlation coefficients between P, CRT and RAp and number of missing values per treatment and treatment*concentration were obtained using the CORR procedure in SAS. Additionally, the average preference for the negative control (water) in each model was compared to the neutral value of 50% using a Student's t test. Results of correlations and comparisons to the neutral value were considered significant at P<0.05. All the statistical analyses were carried out with the statistical package SAS® (version 9.1., Software SAS Institute Inc., Cary NC, 1991-2001).

Individual DOCH model

The mathematical model used was:

$$Y_{ij}= \mu +\alpha_i+\beta_j+(\alpha\beta)_{ij}+\varepsilon_{ij}$$

where Y_{ij} is the dependent variable for the observation; μ is the general mean for all observations; α_i is the fixed effect of treatment; β_j is the fixed effect of sex ;$(\alpha\beta)_{ij}$ is the interaction between treatment and sex; and ε_{ij} is the residual error.

Pair DOCH model

The mathematical model used was:

$$Y_{ij}= \mu +\alpha_i+\beta_j+(\alpha\beta)_{ij}+\varepsilon_{ij}$$

where Y_{ij} is the dependent variable for the observation; μ is the general mean for all observations; α_i is the fixed effect of treatment; β_j is the fixed effect of concentration; $(\alpha\beta)_{ij}$ is the interaction between treatment and concentration; and ε_{ij} is the residual error.

Results and discussion

Some solutions presented precipitation for insufficient solubility: L-Glu (at 0.5, 5, 50 and 500mM), L-Gln (at 500mM), D,L-Met (at 50 and 500mM) L-Trp (at 50 and 500mM) and L-Thr (at 500mM). The pH of amino acids were between 6.0-7.4 at 500mM, 7.0-7.6 at 50mM, 7.4-7.8 at 5mM and 7.6-7.8 at 0.5mM, except for L-Glu that were 3.5, 4.5, 6.0 and 7.1, respectively, water and sucrose solutions were 7.8.

Comparison between models (individual vs. pair)

Behavioural results showed significant differences between models (P<0.05), pigs from the individual model performed more vocalizations (V) and attempts to escape (AS), and a longer latency to start making the choice test (TL) than pigs from the pair model. In the individual model almost 50% of piglets took more than 2 minutes to make a choice in the last day of training for only 13% from the pair model. The V and AS are parameters used to evaluate reactivity to social isolation (Fraser, 1975; Schader and Todt, 1998; Andersen et al., 2000; de Jong et al., 2000; Boissy et al., 2005; Chaloupková et al., 2007) whereas TL measures the adaptation of the piglet to novel conditions, like the test pen, the stainless steel containers and other testing methodological conditions (Andersen et al., 2000; Hillmann et al., 2003; Chaloupková et al., 2007). The TL may be also considered as a parameter to assess the animal operant response to the DOCH procedure. These behavioural parameters are highly correlated with physiological responses of stress (Schader and Todt, 1998; de Jong et al., 2000; Tedo, 2003). On the other hand, the frequency of piglets with a total consumption (TC) less than 50g (the rate 4 in Table 2) during the last training session was 40% and 17.4% in the individual and pair models, respectively (P<0.05). No differences were observed in the average of TC between models, both between 100-200g/pig.

Pig's adaptation to the experimental conditions was ranked through a score, developed from the addition of behavioural and TC parameters (Table 2). The score was also used as a criterion to exclude animals from the DOCH test sessions. Animals that had a high score may be considered those less adapted to social isolation, novel conditions and operant conditioning procedures. Adapted and selected pigs for the experimental sessions were those without AS and V, those that were performing well the double-choice test (TC between 50-500g) even if they lasted more than 30s to approach to the containers (LT). In these conditions, pigs recorded a score level lower than 5. The comparison of the score between models showed that it was higher in animals from the individual model than from the pair model, 4.6 vs. 2.5 (P<0.05).

A total number of 82 pigs were trained individually without a previous adaptation period to social isolation and 96 pigs in pairs. In the individual model, 49 animals were used in test sessions and 33 were excluded because they had a score higher than 5. Excluded animals showed AS, V, TL higher than 2 minutes, and a TC lower than 100g. In the pair model, all trained animals were used in DOCH test sessions. None of the pigs from the pair model had a score higher than 5.

In the individual model, no differences were observed between males and females, but it appeared a significant interaction in terms of preference (P) between sex and the 500mM amino acid solution of Lysine. Females preferred more the L- Lys than males (P<0.05). The individual model did not show differences in P, CRT and RAp between the negative (tap water) and the positive (sucrose, 500mM) controls. Pigs were not able to discriminate between water and sucrose after training. In the pair model, all these parameters showed differences between control solutions (P<0.01). Pigs were able to distinguish between both control solutions after training in pairs. They preferred, consumed and had more appetence for sucrose (500mM) than water (*Table 3*).

Table 3 > Results of positive (sucrose, 500mM) and negative (Tap water) controls in double-choice individual (per pig) and pair (per pen) models.

		INDIVIDUAL				PAIR		
Control	N[1]	P (%) (Mean±SEM)	CRT (g/s) (Mean±SEM)	RAp (g/s) (Mean±SEM)	N[1]	P (%) (Mean±SEM)	CRT (g/s) (Mean±SEM)	RAp (g/s) (Mean±SEM)
Water (-)	20	53.46±2.50	0.85±0.20	0.46±0.05	32	46.14±3.18a	0.76±0.08a	0.35±0.04a
Sucrose (+)	33	76.81±3.60	1.33±0.13	0.95±0.06	70	75.71±2.78b	2.20±0.14b	1.67±0.11b

1 - Number of animals (individual model) or pens (pair model) that reach the minimum total consumption level of 50g/pig the last day of training sessions. a,b Means with different letter are significantly different.(P<0.01).

According to Manteca and Deag (1994), physiological and behavioural responses to stressors may affect experimental results. Since individual animals differ in their pattern of response to stressors, it is suggested that stress during experiments has the potential for increasing variability in responses to experimental treatments. The identification of individual differences in temperament, as the way that animals react to environmental change or challenge, may allow us to reduce the incidence of some welfare problems and the number of animals that need to be used for the experimental procedures (Manteca and Deag, 1993, 1994). The exposure to novelty and social isolation are two potent stressful situations in pigs (Andersen et al., 2000; Hillman et al., 2003). Results showed that animals were better adapted to the operant training

procedure when they were maintained in pairs rather than individually. In fact, in the individual model more training days were needed (4 vs 2 days) to train 50% of the animals ready for carrying out DOCH tests. Moreover, individually tested pigs were not able to clearly distinguish between control solutions (500mM sucrose vs tap water).

We have used an operant conditioning methodology to assess sensory effects associated with preference. The operant conditioning technique tries to teach the animal to "work" for a reward of food or nutrient following a reinforcement schedule with successive approximations to the desired response, thereby establishing the operant behaviour (Arave, 1996; Forbes, 2007). The operant technology is often employed in farm management, like with drinkers in which an animal pushes a valve with its nose or snout (response) to receive water (reinforcement). This methodology has been used several times to obtain quantitative measures of preference for different feeds and nutrients (Kennedy and Baldwin, 1972; Baldwin, 1996; Forbes, 2007). Pigs are very easily conditioned and sugar works very well as reinforcement in operant conditioning techniques (Kennedy and Baldwin, 1972; Baldwin, 1996). Therefore, our training sessions from both DOCH models were based on an operant conditioning schedule, where 500mM of sucrose solution was used as a positive reinforcement. Training sessions in pig DOCH tests have been previously established between 3 and 5 days (Glaser et al., 2000; Tinti et al., 2000; Nofre et al., 2002) with a previous adaptation period to social isolation and/or a fasting period before DOCH testing. Starved pigs are highly motivated to obtain food (Baldwin and Meese, 1979) and they learn very quickly in front of an energetic reward (sugar, source of energy). In our conditions, pigs were maintained under a fed-status since the objective was to learn about the pig amino acid preference, level of consumption and appetence as a criterion of its nutrient sensorial perception. The fed status in our pigs allowed that the motivation for amino acid consumption was not driven by energetic requirements.

Nutrients can trigger a cephalic response under a fed-status through sensory systems (Power and Schulkin, 2008). These cephalic responses are very fast, for example there is a cephalic insulin response within 4 minutes after glucose taste stimulation (Just et al., 2008), therefore, the duration of the DOCH test (2'), the limited amount of amino acid solution offered (250 ml of total solution), and the interval between test sessions (9am and 12pm) diminished the potential influence of postingestive factors on the ingestive behaviour (Glaser et al., 2000; Laska et al., 2008) and the effect of cephalic phase responses.

The evaluation of behavioural and consumption parameters through the described score showed that it is possible to carry out DOCH tests individually without a previous time for adaptation to social isolation in pigs under a fed-status, but it may be necessary to invest more time to habituate animals to the test procedure or, alternatively, it may be required to select animals before starting test sessions to prevent stressful factors that may influence the experimental results. Results of preference tests were similar in both models (Figure 1), but maintaining pigs in pairs made adaptation to operant conditioning easier, allowed to reduce training time and avoided exclusion of animals for inappropriate testing performance.

Results of Double-choice tests

No differences in Preference (P, %), rate of consumption of test solution (CRT, g/s) and rate of appetence or preference (RAp, g/s) were showed due to the order of tested amino acid solutions, in terms of DOCH testing session (at 9a.m. or 12p.m.) and day of testing (within 5 days). The P, CRT and RAp values were used to assess the ability of pigs to discriminate among different amino acids and different concentrations and to describe their attraction to consume them. The RAp was born from combining the P and the CRT values obtained in DOCH testing sessions. Schwartz et al. (2009) compared taste acceptance in humans using also two measures: ingestion and liking of test solutions. These authors considered these measures complementary to clarify conflicting results obtained with only one measure due to the high variability in taste acceptance among individuals. We observed a high variability in CRT among individuals and amino acid solutions. Initially the RAp was created trying to normalize data obtained from P taking into account this CRT variability. For example, we can have two pigs showing a 70% of P for the same amino acid solution, but pig 1 consume totally 100g (70g of test solution) and pig 2 consume totally 300g (210g of test solution) during the DOCH test session. The RAp for pig 1 is 0.4g/s and for pig 2 is 1.2g/s. We may consider that pig 2 has a higher appetence for the amino acid solution than pig 1. Another usual situation, the same pig with 60% of preference for the solution 1 and total consumption of 300g (180g of test solution) and 80% of preference for solution 2 and total consumption of 100g (80g of test solution). The RAp value for solution 1 is 1.1g/s and for solution 2 is 0.5g/s. According to Nielsen (1999), it is assumed that on a given food the animal has a preferred feeding rate; therefore, we may consider that this pig is more attracted to consume solution 1. Looking at our results, we may define the rate of appetence or preference (RAp, g/s) as the rate of consumption of an offered test solution that pigs in a fed-status would be predisposed or attracted to consume during a two-minute period (test session) without having to choose. Our results of RAp will need further studies to demonstrate

that individual pigs fed ad *libitum* would maintain the same rate of consumption for a determined solution as we tried to predict with our RAp parameter.

Pigs showing a total consumption lower than 50g were considered to not perform the double-choice test. The quantity of 25g/container (test and control) was the minimum level of consumption established for including data into the analysis. The accuracy of the scale was 10g and results from preliminary studies showed loses of approximately 15g not related to direct consumption (pigs putting their feet in containers or moving the containers with their snouts). These animals that did not reach the minimum total consumption were considered as missing values. The number of missing values was numerically higher in the individual model than in the pair model, particularly for less preferred amino acid solutions such as 500mM L-Trp (Table 4). Low levels of P, CRT and RAp correlated negatively (r= -0.375; r=-0.489 and r=-0.387, respectively; P<0.001) with the number of animals or pens that did not reach a minimum total consumption rate of 0.42g/s per pig (missing values).

Table 4 > Percentage of animals that did not reach a minimum total consumption of 50g/pig in individual and a pair DOCH model, referred to as "% of missing values".

INDIVIDUAL MODEL		PAIR MODEL	
Nutrient[1]	% of missing values[2]	Nutrient[1]	% of missing values[2]
D,L-Met	40.00	D,L-Met	20.00
L-Thr	ND	L-Thr	18.75
L-Lys	14.50	L-Lys	9.09
L-Trp	30.70	L-Trp	9.09
Gly	30.00	Gly	16.65
L-Gln	16.67	L-Gln	21.25
L-Glu	10.34	L-Glu	2.08
MSG	8.00	MSG	0.00
L-Ala	ND	L-Ala	8.33
Water (-)	40.00	Water (-)	52.25
Sucrose (+)	15.52	Sucrose (+)	10.41

1 - All amino acid solutions were tested at 500mM, water as a negative control and sucrose as a positive control.

2 - ND were solutions not tested in the individual model.

Differences among amino acids were found in both models for all the parameters evaluated (P<0.001). It was also showed differences in the same amino acid depending on its concentration (P<0.01). The L-Thr (500mM) and L-Trp (50 and 500mM) at high concentrations had less P, less CRT and less ARp than at low concentrations (*Figure 1*).

Furthermore significant interactions in P were found between amino acid and sex (P<0.05) in the individual model and in P, CRT and RAp between amino acid and concentration (P<0.01) in the pair model. Females showed a higher preference for L-Lys than males. Owen et al. (1994) showed differences in self-selection and performance of growing pigs due to Lys dietary content depending on sex. Gilts chose a higher-lysine diet than barrows because gilts required higher lysine contents than barrows to maximize their growth.

Figure 1 > Comparisons among concentrations (5, 50 and 500mM) of the same amino acid (Mean±SEM) of a) P (%), b) CRT (g/s) and c) RAp (g/s). Data from the pair DOCH model (2 pigs/pen).
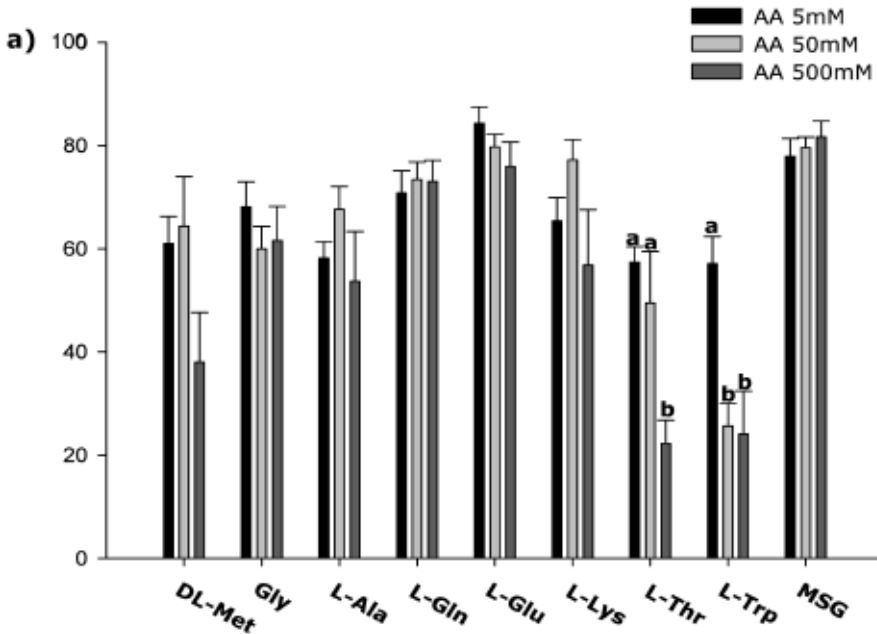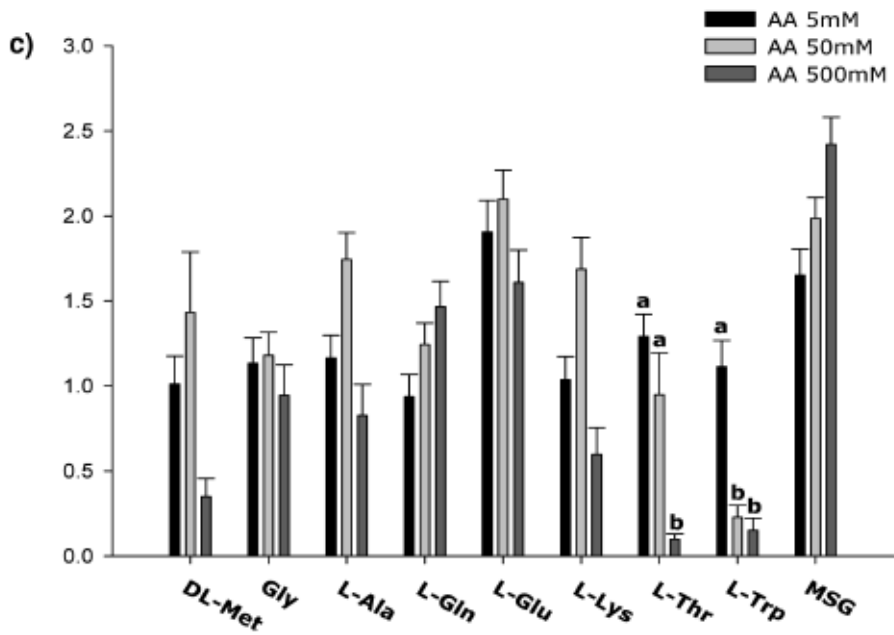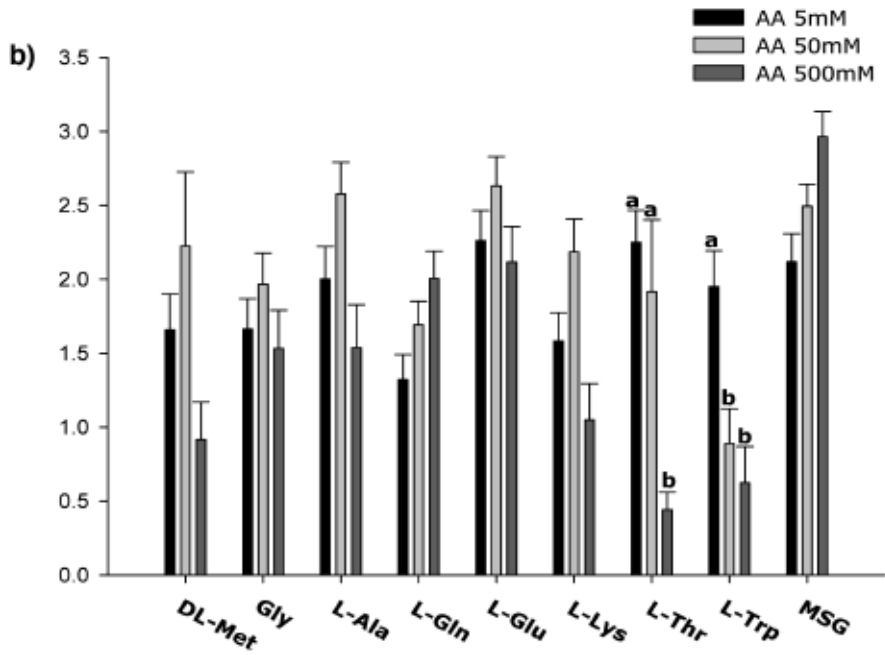
Results of tested amino acids at 500mM in individual and pair DOCH models revealed that MSG and L-Glu had the highest P, CRT and RAp, and the opposite applies to L-Trp (*Figure 2*). The P for the negative control (water) was not different from a neutral value of 50% in both models; therefore, water P, CRT and RAp values were taken as reference values of neutrality. Although as it was mentioned before, in the individual DOCH model it was not showed differences between both control solutions (500mM sucrose vs. tap water).

Doses of 500mM were initially chosen to elicit strong responses. This dose was extremely high compared to physiological amino acid levels. Concentrations of amino acids vary greatly between tissues and physiological conditions, although in average, the majority of plasma concentrations of essential (EAA) and non-essential amino acids (NEAA) have been shown to be between 0.5 to 50mM. For example L-Gln is between 0.5 to 1mM and L-Lys is around 0.5mM. In other tissues, EAA and NEAA levels have been described around 5 to 50mM. For example, L-Gln in the skeletal muscle of the pig is between 5 to 20mM and in a 25kg pig whole body L-Lys level is around 50mM (Simon, 1989; Kim et al., 2007). Taking into account these concentrations, an amino acid dose-response (0.5, 5, 50 and 500mM) was performed in the pair DOCH model.

Tested EAA were those commonly used to supplement pig starter diets, L-Lys (HCl), D,L-Met, L-Thr and L-Trp and tested NEAA were those described as umami tastants in other mammals such as L-Glu and its salt (MSG) (Li et al., 2002; Nelson et al., 2002; Bachmanov and Beauchamp, 2007; Roura et al., 2008), and others very abundant as free amino acids in the body such as L-Gln, L-Ala and Gly (Waterlow and Fern, 1983). The lowest dose of 0.5mM was only tested for MSG, L-Gln, L-Ala, Gly, L-Lys and L-Trp. The L-Glu, D,L-Met and L-Thr were not tested at that concentration.

Figure 2 > Results (Mean±SEM) of P (%), CRT (g/s) and RAp (g/s) for 500mM amino acid solutions in both models, sucrose (500mM) as positive control and tap water as a negative control. Data from the individual model corresponds to 1 pig; data from the pair model corresponds to two pigs per pen.
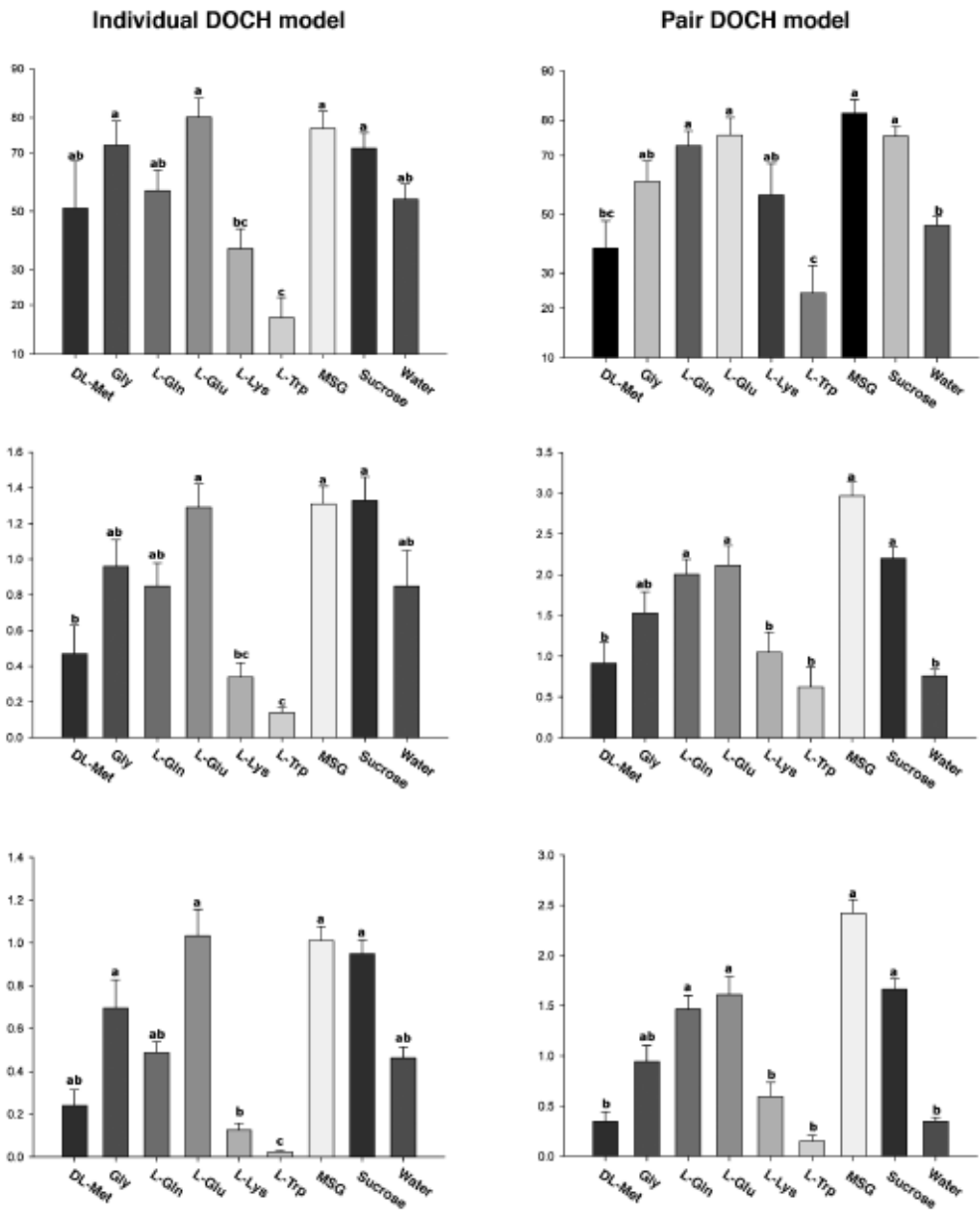
Different letters mean values significantly different (P<0.01).

The P, CRT and RAp results of all tested amino acids showed that pigs under a fed-status preferred and had a high appetence for some NEAA at high concentrations, especially those previously reported as umami tastants in rodents, humans and potentially in pigs such as MSG, L-Glu and L-Gln (Danilova et al., 1999; Bachmanov and Beauchamp, 2007; Roura et al., 2008) (*Tables: 5, 6 and 7-marked in bold*). Responses between MSG and L-Glu were very similar in non-buffered solutions and independently on their pH (acid –pH=3.50- and neutral –pH=7.2- for the L-Glu and MSG solutions, respectively) and the salty taste of MSG. Sucrose (positive control) elicited the same taste response as L-Gln, L-Glu and MSG at the same concentration (500mM). Sucrose has been described as a pleasant tastant for pigs (Baldwin, 1996), therefore, we suggest L-Gln, L-Glu and MSG as pleasant compounds as sucrose like was previously described in other species (Bellisle, 1999) (*Tables: 5, 6 and 7*).

The other NEAA (Gly, L-Ala) may be also considered as umami tastants, although they seem to elicit sweet taste cues in humans and rodents (Bachmanov and Beauchamp, 2007; Roura et al., 2008). Pigs showed a weak preference for L-Ala and Gly at all concentrations, but they consumed and had an appetence for them at 5 and 50mM similar to MSG (*Tables: 5, 6 and 7*). Danilova et al. (1999) showed that Gly could elicit responses in bitter taste nerve fibers at high concentrations which could explain lower responses compared to those obtained from MSG and L-Glu.

The EAA solutions at 500mM were the least preferred, consumed and appetent to pigs (*Tables: 5, 6 and 7*). Pigs showed a strong aversion for L-Thr and L-Trp at the highest concentration. The D,L-Met and L-Lys were close to neutrality. The responses of preference to EAA were dose-dependent (*Figure 3*). The EAA solutions in the physiological range (0.5, 5 and 50mM) had a preference, consumption and appetence close to the neutral value (*Tables 5, 6 and 7*). Pigs only showed preference for one EAA, L-Lys at 50mM, being as preferred as other potential umami tastants (MSG, L-Glu and L-Gln) (*Table 5*).

Edmonds et al. (1987) studied the effect of excess levels (4%) of D,L-Met, L-Trp, L-Arg, L-Lys or L-Thr on growth and dietary choice in the pig. They observed that pigs clearly preferred a control diet over any of the amino-acid imbalanced diets, especially with L-Trp, and also that an excess of those depressed growth after only 1 day on test. The excess of D,L-Met and L-Trp were the most growth-depressing amino acids in the pig. Bradford and Gous (1991) concluded that pigs on a choice between deficient and excessive protein content chose diets that maximized performance and carcass leanness and matched amino acid intake to requirements. Pigs trend to consume more L-Lys than need for maximum performance (Henry, 1987; Owen et al., 1994). The L-Lys is the most limitant amino acid in pigs. The previous comments may

explain part of our results with L-Lys and the other amino acids at physiological concentrations (0.5, 5 and 50mM). Our pigs were in the middle of their growing stage were their requirements for EAA are normally high (NRC, 1998). These results may demonstrate certain awareness for amino acids in pigs where taste cues may help to recognize them. On the other hand, the evolution of these preferences, rates of consumption of test solution and appetence for them could change during time due to possible postingestional effects (Ettle and Roth, 2004, 2005; Roth et al., 2006). Concentrations of EAA increase during the postabsorptive phase, reach the maximum in pigs at approximately 2 hours after feeding, and return to fasting levels 10-15h later (Simon, 1989).

Looking at the lowest tested concentration (0.5mM), pigs were not able to detect differences in taste cues of the EAA (L-Lys and L-Trp) and NEAA (Gly, L-Ala, L-Gln and MSG) at this level compared to water (*Table 6*). Pigs only showed a high CRT in the L-Ala solution, but their preference and appetence for this amino acid solution was the same as in the other solutions and controls (*Table 5 and 7*). Therefore, we may determine that a possible threshold of detection of these amino acids is above 0.5mM (*Figure 3*).

Table 5 > Pair DOCH model results (Mean±SEM) of Preference (%) for different concentrations of amino acids, sucrose (500mM) as the positive control and tap water as the negative control.

	TESTED CONCENTRATION (mM)*										
	0.5			**5**			**50**			**500**	
AA	**Mean±S.E.M**			**Mean±S.E.M**			**Mean±S.E.M**			**Mean±S.E.M**	
DL-Met	ND			60.99	±5.26	abcd	64.38	±9.58	abc	38.07	±9.60	bc
Gly	42.97	±10.71	ab	68.15	±4.82	abcd	59.96	±4.41	abc	61.60	±6.64	ab
L-Ala	67.71	±7.03	ab	58.17	±3.19	c	67.65	±4.46	ac	53.74	±9.62	bc
L-Gln	53.43	±4.87	ab	70.84	±4.31	abc	73.43	±3.37	ac	73.03	±4.13	a
L-Glu	ND			84.30	±3.23	b	79.76	±2.45	a	75.97	±4.68	a
L-Lys	49.94	±6.73	ab	65.42	±4.51	abcd	77.25	±3.91	a	56.84	±10.73	abc
L-Thr	ND			57.37	±3.04	cd	49.46	±10.05	c	22.27	±4.54	c
L-Trp	46.19	±7.63	ab	57.07	±5.28	d	25.64	±4.40	d	24.13	±8.30	c
MSG	52.47	±4.96	ab	77.90	±3.50	ab	79.60	±2.03	a	81.66	±3.09	a
Sucrose	75.71	±2.78	a	75.71	±2.78	ab	75.71	±2.78	a	75.71	±2.78	a
Water	46.14	±3.18	b	46.14	±3.18	c	46.14	±3.18	b	46.14	±3.18	b

* Values in the same column with different letters are significantly different (P<0.01). ND=not determined.

In rats, different thresholds for some of these amino acids were determined and were also above to this level, for example, the threshold for Gly was between 5-25mM (Pritchard and Scott, 1982; Delay et al., 2007). Tinti et al. (2000) also showed in pigs a low consumption or no consumption of Gly, L-Ala, L-Gln and L-Thr solutions between 60-120mM. Studies to determine MSG and NaCl detection thresholds in non-human primates (Laska et al., 2008) showed that non-human primate species with a higher content of animal protein in their diet had a lower threshold for MSG (around 40mM). The MSG is believed to indicate the presence of dietary animal protein (Ninomiya, 1998). This threshold was lower than in humans (Laska and Hernández-Salazar, 2004). Our data showed that MSG and L-Glu threshold in pigs may be below 5mM considering that the protein content in our piglet diet is high (*Table 1*). Therefore, we may suggest higher sensitivity of piglets to detect lower MSG and L-Glu concentrations than humans and non-primate humans.

Table 6 > Pair DOCH model results (Mean±SEM) of Rate of consumption of test solution (g/s) for different concentrations of amino acids, sucrose (500mM) as the positive control and tap water as the negative control.

	TESTED CONCENTRATION (mM)*										
	0.5			**5**			**50**			**500**		
AA	**Mean±S.E.M**			**Mean±S.E.M**			**Mean±S.E.M**			**Mean±S.E.M**		
DL-Met	ND			1.66	±0.24	ab	2.23	±0.50	abc	0.92	±0.25	abd
Gly	0.82	±0.10	ab	1.66	±0.21	b	1.97	±0.21	ac	1.53	±0.26	abc
L-Ala	1.73	±0.40	a	2.00	±0.22	a	2.58	±0.21	ac	1.54	±0.29	abc
L-Gln	1.10	±0.30	ab	1.32	±0.17	ab	1.69	±0.16	a	2.01	±0.18	ac
L-Glu	ND			2.26	±0.21	a	2.63	±0.20	c	2.12	±0.24	ac
L-Lys	1.17	±0.27	ab	1.58	±0.19	ab	2.18	±0.22	ac	1.05	±0.24	abad
L-Thr	ND			2.25	±0.22	a	1.92	±0.49	abd	0.44	±0.12	d
L-Trp	1.17	±0.29	ab	1.95	±0.24	a	0.89	±0.24	d	0.63	±0.24	d
MSG	1.12	±0.44	ab	2.12	±0.19	a	2.49	±0.15	c	2.97	±0.17	c
Sucrose	2.20	±0.14	a	2.20	±0.14	a	2.20	±0.14	ac	2.20	±0.14	ac
Water	0.76	±0.08	b	0.76	±0.08	b	0.76	±0.08	bd	0.76	±0.08	b

* Values in the same column with different letters are significantly different (P<0.01). ND=not determined.

Table 7 > Pair DOCH model results (Mean±SEM) of Rate of Appetence (g/s) for different concentrations of amino acids, sucrose (500mM) as the positive control and tap water as the negative control.

TESTED CONCENTRATION (mM)*											
	0.5			**5**			**50**			**500**		
AA	**Mean± S.E.M**			**Mean±S.E.M**			**Mean±S.E.M**			**Mean±S.E.M**		
DL-Met	ND			1.01	±0.16	ab	1.43	±0.36	ab	0.35	±0.11	abd
Gly	0.35	±0.17	ab	1.13	±0.15	a	1.18	±0.14	a	0.94	±0.18	ab
L-Ala	1.17	±0.22	ab	1.16	±0.13	a	1.74	±0.16	a	0.83	±0.18	ab
L-Gln	0.59	±0.16	ab	0.94	±0.13	ab	1.24	±0.13	a	1.46	±0.15	ac
L-Glu	ND			1.91	±0.18	a	2.10	±0.17	a	1.61	±0.19	ac
L-Lys	0.58	±0.15	ab	1.03	±0.14	a	1.69	±0.19	a	0.60	±0.16	abd
L-Thr	ND			1.29	±0.13	a	0.95	±0.25	a	0.10	±0.03	d
L-Trp	0.54	±0.13	ab	1.11	±0.15	a	0.23	±0.07	c	0.15	±0.07	d
MSG	0.59	±0.11	ab	1.65	±0.15	a	1.99	±0.12	a	2.42	±0.16	c
Sucrose	4.05	±0.27	a	1.67	±0.11	a	1.67	±0.11	a	1.67	±0.11	a
Water	0.35	±0.04	b	0.35	±0.04	b	0.35	±0.04	bc	0.35	±0.04	b

* Values in the same column with different letters are significantly different (P<0.01). ND=not determined.

In mice, the umami taste receptor T1R1/T1R3 function as a broadly tuned sensor responding to most of L-amino acids (not to their D-enantiomers) and it is enhanced by nucleotides whereas in humans preferentially respond to L-Glu and L-Asp. Some L-amino acids were described as sweet in humans such as L-Ala, L-Gln, L-Thr, L-Arg, L-Pro and Gly but in mice they are considered umami tastants (Li et al., 2002; Nelson et al., 2002; Zhao et al., 2003; Manita et al., 2006; Bachmanov and Beauchamp, 2007, 2008). Therefore, apart from differences in threshold detections, there are more evidences of different taste perception among species.

Figure 3 > Dose-response (0.5, 5, 50 and 500mM) evolution of pig preferences for several amino acids (essential: L-Lys and L-Trp, and non-essential: L-Ala, Gly, L-Gln and L-Glu-Na or MSG) depending on their concentration.
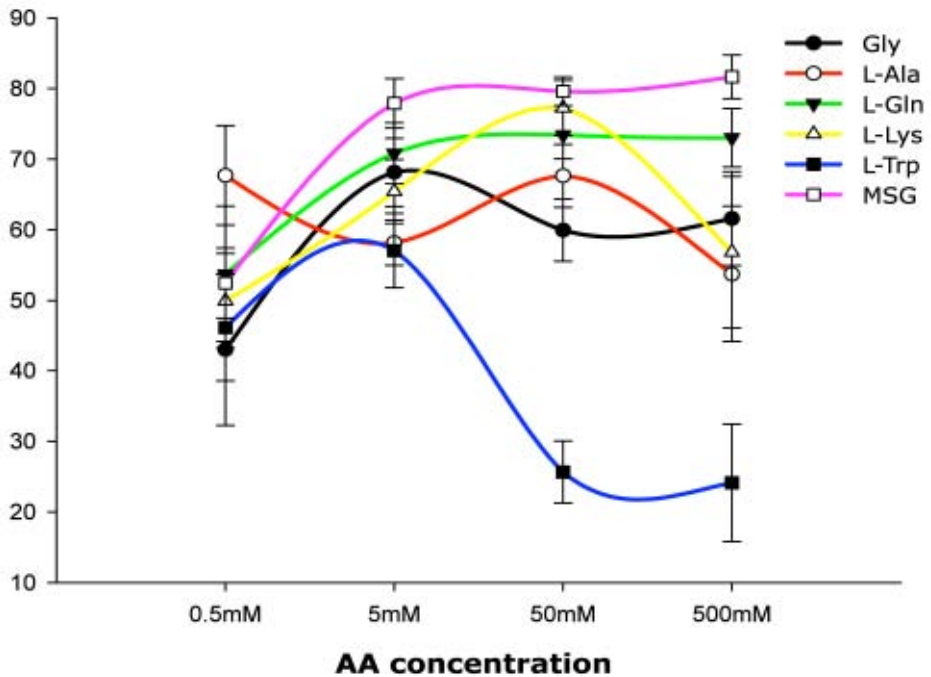
Most studies referring to palatability and appetite in pigs have been expressed as the % of preference (Kennedy and Baldwin, 1972;Seabolt et al., 2008; Solà-Oriol, 2008). The DOCH tests were also used to study dietary choice depending on specific EAA (L-Lys, L-Trp, L-Thr and D,L-Met) but accounting for the total consumption of the test diet (Owen et al., 1994; Kirchgessner et al., 1999; Ettle and Roth., 2004, 2005; Roth et al., 2006). Other DOCH tests were focused on pig gustatory responses for Gly and L-AA considered effective those with 80% or more of the tested solution consumed and the volume of water simultaneously presented unchanged (Tinti et al., 2000). In our choice-feeding results, we used the percentage of preference, the rate of consumption of test solution and our developed rate of appetence or preference to take into account both the hedonistic incentive (preference) but also the motivation of the animal to consume a specific amino acid solution (appetence). Pigs move away after an initial sampling and the total and test consumption are very low when they do not have any interest in the solutions (Glaser et al., 2000; Tinti et al., 2000). Pigs that did not reach a minimum rate of total consumption in our DOCH tests were

considered missing values for this amino acid concentration. We showed that less preferred, consumed and less appetent amino acid solutions had a high number of missing values (*Table 4*).

Looking at comparisons among taste perception for different amino acids in rodents, it has been showed that L-Ala elicited taste qualities nearly identical to MSG at low concentrations when the effect of sodium was neutralized through the Na+ channel blocker amiloride (Taylor- Burds et al., 2004). In rats, the taste profile of MSG (with amiloride) compared to four amino acids that differed in their side-chain properties (L-Pro, L-Ser, L-Arg and Gly) elicit similar taste responses (Delay et al., 2007). In terms of preference, our results showed similarities among MSG, L-Glu and L-Gln, being stronger at high concentrations. The responses for Gly and L-Ala were quite different, having in general low levels of preference compared to them (*Table 5*). Therefore, we may suggest different sensors in pigs involved in detection and identification of these amino acids.

Studies in mice using a 48-h two-bottle test, showed that L-Ala, L-Thr and L-Gln intakes and preferences significantly increased with increasing solution concentrations (from 1 to 300mM). It seemed that this response was independent on if they were EAA or NEAA. Their preference and consumption were considered for their sweetness, although a 48-h continuous nutrient consumption like amino acids could have influenced their response due to postingestional effects (Bachmanov and Beauchamp, 2008). Our results showed differences in pig preferences, consumptions and appetencies between L-Thr, L-Ala and L-Gln at the highest tested concentration (500mM). Pigs showed a significantly lower preference, consumption and appetence for the EAA (L-Thr) than for the NEAA (L-Ala and L-Gln) (*Tables: 5, 6 and 7*). On the other hand, fasted pigs strongly rejected to consume L-Trp solutions even at a low concentration (14.6mM) (Tinti et al., 2000). Our L-Trp solutions were also strongly rejected by pigs in a fed-status at 50 and 500mM. In general, our pig preferences, consumptions and appetencies for EAA were lower when concentrations were increased. In the NEAA solutions they normally increased as concentrations were also increased, especially in L-Gln, L-Glu and MSG (*Tables: 5, 6 and 7*).

Another way to evaluate gustatory stimulation in pigs is through electrophysiological recordings of whole or single fibers of the chorda tympani nerve (CT- innervating fungiform taste buds) and glossopharyngeal nerve (GP- innervating circumvallate taste buds) responses during taste stimulation (Danilova et al., 1999; Hellekant and Danilova, 1999). These studies were done with 1 to 7 week-old piglets and showed that CT nerve exhibited large responses to MSG in "salty" and "sweet" fibers than GP

nerve, although responses to MSG were not attributable only to sodium ions, since NaCl (100mM) gave a smaller response in CT nerve. The presence of nucleotides (3mM IMP and GMP solutions) did not affect the response of 70mM MSG or 400mM Gly in contrast with the current understanding in other animal species (Kawamura and Kare, 1987). Moreover, Danilova et al. (1999) also showed that Gly had the highest response in 14 day-old pigs and the lowest in 7 week-old pigs stimulating "bitter", "sour" and "sweet" taste nerve fibers. Our pigs showed weak responses with Gly, maybe due to their age and, as it has mentioned before, to the stimulation of these fibers that cause rejection. As previously observed with other pig *in vivo* data (Tinti et al., 2000), we may suggest that pig electrophysiological recordings from CT and GP nerves may also corroborate our behavioural data.

Several studies demonstrated that choice-feeding was not only evoked by diets varying in their overall protein content (Kyriazakis and Emmans, 1990), amino acids such as L-Lys, L-Trp, L-Thr or D,L-Met also played a role in dietary choice in piglets (Owen et al., 1994 – L-Lys; Ettle and Roth., 2004, 2005 –L-Trp and L-Thr; Roth et al., 2006 – D,L-Met). Pigs given a choice of diets deficient or adequate in Lysine (Lys) showed a specific preference for Lys because they were able to detect metabolic changes and modify their feed intake pattern to correct possible imbalances (Kirchgessner et al., 1999). The same was observed in piglets that select a diet more adequate in L-Trp over a L-Trp-deficient diet (Ettle and Roth, 2004), having stronger responses than those showed in L-Lys and D,L-Met experiments (Roth et al., 2006). When given a choice, piglets preferred a diet better balanced in these EAA. Ettle and Roth (2004) observed a spontaneous preference for diets containing a high level of L-Trp at the third week of the experiment, where animals made a choice in only 5 minutes, presumably due to the association of dietary taste and odor cues with postingestive consequences. Therefore, dietary selection seems to be based on a learning process, and piglets need a period of time to associate the sensory cues of feeds with their physiological impact.

In humans and rats L-Glu and MSG have been described as umami tastants and purine nucleotides as umami enhancers, both increasing food palatability and stimulating food consumption (Yoshii et al., 1986; Bellisle, 1999). According to Birch (1999) there is an innate predisposition to prefer or reject basic tastes, although those may be altered via experience with food and eating. Our data showed that umami may be one of the basic tastes that weaned pigs had predisposition to prefer under a fed-status. The stimulation of feed intake using potential umami tastants may be a tool for increasing diet consumption, especially during critical periods of nutritional stress

such as weaning. The trend in piglet diets is to reduce their protein content and supplement them with synthetic amino acids for environmental and healthy purposes. Potential umami responses may help to recognize sources of animal protein and to stimulate their feed intake, always taking into account that these diets should be balanced to avoid postingestional effects that can reduce their consumption.

In summary, our pigs under a fed-status preferred, consumed and showed a high appetence for NEAA solutions of known umami tastants in humans and rodents and avoided EAA solutions at high concentrations. Pig responses were variable depending on whether the tastants were EAA or NEAA. The L-Lys (HCl) was the only EAA significantly preferred, consumed and appetent at physiological concentrations. Our pigs were in a growing stage and it may explain an innate predisposition to prefer the most limiting amino acid such as L-Lys in a cereal-soy based diet (Owen et al., 1994; Williams et al., 1997). We may suggest that pigs are able to differentiate among different concentrations of EAA and they have an innate rejection to consume EAA at concentrations that could trigger an undesirable postingestional effect like a dietary amino acid imbalance. In NEAA, L-Ala and Gly showed pig responses lower than sucrose presumably due to their fed-status and testing age (6-7 week-old). Pigs, in turn, showed a high preference, consumption and appetence for potential umami tastants, L-Glu and MSG. The L-Gln showed more similar results to L-Glu and MSG solutions than other sweet amino acids like Gly and L-Ala, suggesting that it may be considered as umami rather than sweet. Moreover, our pigs were able to discriminate amino acid solutions at concentrations higher than 0.5mM. The sensitivity of pigs to these solutions was higher than in other omnivores such as humans, non-human primates and rodents.

The porcine T1r1/T1r3 stimulation by L-amino acids and Gly, and if they are triggering the same afferent pathways that control the voluntary feed intake as in other species, merits further study. It would be useful as well to test these amino acids combined with purine nucleotides (IMP, GMP). Nucleotides may potentiate the amino acid preference and appetence responses such as in human or rodents (Kawamura and Kare, 1987). Moreover, it is important not only to differentiate between sweet and umami tastants, but also to know these amino acids with aversive tastes (bitter), potentially L-Trp and L-Lys (HCl), that could negatively affect feed consumption (Bachmanov and Beauchamp, 2007). Finally, further studies should be done looking at the postingestional effects and learning process of pigs when these amino acids are administrated continuously.

Conclusions

The pair model developed in this work may be more appropriate to study pig preferences and appetence for amino acid solutions or other nutrients. It prevents the social isolation effect on pig learning capacity for DOCH testing and allows reducing the training period. The individual 2-minute DOCH test model avoiding an adaptation period to isolation might be adequate if animals are previously selected based on their behaviour and consumption and if its time on training is more than only 4 days.

Weaned pigs under a fed-status were able to discriminate solutions of amino acids except when those were offered at 0.5mM. In general, piglets showed significant preferences for NEAA with a higher appetence for potential umami tastants at high concentrations (MSG, L-Glu and L-Gln). However, this taste response changed with EAA, resulting in significant aversions at high concentrations (L-Trp, L-Thr).

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Chapter 2

L-amino acids highly preferred by pigs in a fed state are agonist ligands to a porcine T1r1/T1r3 expressing cell reporter system [1,2]

Chapter 2 >>>>>>>>

Abstract

The umami taste is a pleasant taste related to protein born nutrients such as amino acids and oligopeptides. In humans and rodents, *in vitro* studies showed that the heterodimer T1R1/T1R3 is stimulated by L-amino acids and its response is enhanced by nucleotides. This umami taste receptor displays selectivity and sensitivity differences that mimic amino acid taste differences between rodents and humans. Our *in vivo* data showed high preferences and appetencies in pigs for potential umami tastants such as monosodium glutamate (MSG), L-Glutamic acid (L-Glu), L-Glutamine (L-Gln), L-Alanine (L-Ala) and Glycine (Gly). Nevertheless, to describe the umami nature of their taste sensation requires showing the specificity of these ligands for the pig umami receptor. Unfortunately, the pig genome is not still available. The objectives of this study were (1) to identify and characterize the putative umami porcine gene sequences (the pT*as*1r1 and pT*as*1r3), (2) to develop a stable cell reporter system expressing the porcine heterodimer (pT1r1/pT1r3) in order to identify potential ligands amongst Gly and L-amino acids at physiological concentrations (0.5, 5 and 50mM), and (3) to compare their in vitro response to our behavioural data. The porcine umami taste receptor has been identified from vallate papilla of a 6-month-old male pig. The two pig umami genes (pT*as*1r1 and pT*as*1r3) have lower homologies with humans and rodents than with carnivores (i.e. cat) what may explain differences in taste perception. A cell reporter system expressing the pT1r1/pT1r3 receptor was developed for *in vitro* amino acid testing. The cell system showed significant responses to MSG, L-Glu, L-Gln, L-Ala, L-Asn and Gly at all tested concentrations. Finally, our *in vivo* data was significantly correlated to our *in vitro* results meaning that umami agonists are highly preferred by pigs. Therefore, umami tastants could be used to develop new feeding strategies to increase voluntary feed intake in critical stages of pig production, such as at weaning.

Introduction

The sense of taste gives animals the ability to evaluate what they eat and drink ensuring the ingestion of nutritious substances while preventing the consumption of potentially harmful foods (Forbes, 2007). The umami taste (Ikeda, 1909) is considered as a pleasant taste (Bellisle, 1999) mainly related to protein-born compounds such as L-amino acids (AA) and their salts (e.g. such as monosodium glutamate –MSG-) and peptides, enhanced by 5'-ribonucleotides such as inosine (IMP) and guanosine-5' (GMP) monophosphates (Li et al., 2002; Nelson et al., 2002; Ninomiya, 2002). Amino acid gustatory responses *in vivo* (Tinti et al., 2000) or electrophysiological recordings of chorda tympani and glossopharyngeal nerves (Danilova et al., 1999) suggest that umami taste is also present in pigs. Umami compounds such as MSG alone or in combination with nucleotides elicited a strong response in both taste nerves and some of them gave a hedonic response in pigs such as Gly, L-Glu, L-Thr and L-Ala. Our behavioural studies (Chapter 1) showed that potential umami tastants were highly preferred by pigs such as L-Glu (and its salt, MSG) and L-Gln.

The heterodimer T1R1/T1R3, also known as the umami taste receptor (Li et al., 2002; Nelson et al., 2002), is a G-protein coupled-receptor (GPCR) from the T1R family that functions as an L-AA sensor in taste buds of the tongue and soft palate in mammals (Hoon et al., 1999). The T1R1/T1R3 is coexpressed with the α-subunit of the G protein gustducin (Gα), a taste–specific signalling molecule (Wong et al., 1996; Nelson et al., 2001, 2002; Ruiz et al., 2003; Scott, 2005; Sugita, 2006). Mice lacking Gα showed reduced responses to umami tastants, suggesting that Gα plays a role in taste transduction (Wong et al., 1996; Caicedo et al., 2003; Zhang et al., 2003; He et al., 2004). The Gα have other two subunits more, β (Gβ) and γ (Gγ), that are responsible of the activation of the signal transduction molecule phospholipase C (PLCβ2) (Rossler et al., 1998; Huang et al., 1999). The stimulation of the umami taste receptor activates the PLCβ2-dependent pathway through the Gα leading to a release of calcium from intracellular stores. This intracellular calcium release activates another signal transduction molecule called the transient receptor potential M5 ion channel (TRPM5) and results in the entry of monovalent cations (Na^+), membrane depolarization and generation of action potential of taste cells (Adler et al., 2000; Dulac, 2000; Mombaerts, 2000; Firestein 2001; Montmayeur et al., 2001; Li et al., 2002; Nelson et al., 2002; Liu and Liman, 2003; Matsunami and Amrein, 2003; Prawitt et al., 2003; Sugita, 2006; Yasuo et al., 2008).

The identification of new ligands for GPCRs such as the umami taste receptor is facilitated by high throughput screening assays (HTS) based on cell reporter systems that allow to measure receptor responses through second messenger signals such as intracellular Ca^{+2} variations (Hanson, 2006; Nanda et al., 2009). In mice, such a ligand screening assay identified L-AA as umami tastants. Nelson et al. (2002) used a cell reporter system where candidate receptors, promiscuous G proteins and heterodimeric mouse T1R1/T1R3 or the monomeric counterparts T1R3 and T1R1, were expressed in human embryonic kidney (HEK) cells and assayed for stimulus that evoked changes in intracellular Ca^{+2} levels. In this system, the release of Ca^{+2} from internal stores was monitored at the single-cell level using calcium-indicator dyes. Their results showed that responses were dependent on the combined presence of T1R1 and T1R3 and were very selective for L-AA, enhanced by IMP, and without significant responses to D-AA.

According to Zhao et al. (2003), the T1R1/T1R3 receptor display selectivity and sensitivity differences that mimic amino acid taste differences between rodents and humans, whereas the human receptor is more sensitive to L-Glu the rodent receptor have approximately equal sensitivity to L-Glu and the other L-AA (Li et al., 2002; Nelson et al., 2002). Due to differences observed in umami taste perception among species, it is necessary to study the specific ligand-affinity of the porcine T1R1/T1R3 receptor. Therefore, the identification of the porcine umami receptor is required. Unfortunately, the pig genome is being sequenced and is not still available (web pages: *http://piggenome.org* of the International Swine Genome Sequencing Consortium or *http://sanger.ac.uk/Projects/S_scrofa* of the Wellcome Trust Sanger Institute).

The objectives of this study were to identify and characterize the porcine T*as*1r1 and T*as*1r3 gene sequences in pigs, to construct a stable cell reporter system expressing the porcine umami taste receptor (pT1r3/pT1r1), to measure the cell responses to amino acids at physiological concentrations (0.5, 5 and 50mM) and to compare these cell response to the pig *in vivo* preference results.

Materials and methods

Identification and Characterization of pT*as*1r1 and pT*as*1r3

The work was developed in the Animal Science Dpt. of the University of California, Davis (USA) using circumvallate papilla tissue samples obtained from a 6-month-old male pig. The total RNA was extracted using RNeasy fibrous tissue mini kit (Qiagen 74704). Concentration and quality of RNA were determined by optical density measurement and agarose gel electrophoresis. Three µg of RNA was subsequently reverse transcribed using random hexanucleotide primers and SuperScript III enzyme all part of GeneRacer kit (Invitrogen L1500-01). A 200bp porcine expressed sequence tag (EST) with high homology to human T*as*1r3 was located in a public domain library (pig ESTs database from Iowa State University). Gene specific primers were made and the RACE PCR technique was used to obtain cDNA for adjacent 3' and 5' regions of the T*as*1r3 sequence. Candidate PCR products were sequenced and the RACE process was repeated until the full length mRNA sequence was determined.

We were unable to locate a porcine homologue to mouse or human T*as*1r1 in public data bases. Thus, to obtain the pig T*as*1r1 receptor sequence degenerate PCR primers (DP) were designed that covered areas of high homology to the mouse, human, and/or cat genes using Consensus-Degenerate Hybrid Oligonucleotide Primers (CODEHOP) strategy. Primer pairs were used to amplify pig mRNA using relaxed PCR conditions. Eventually primer sets were found that amplified a portion of the T*as*1r1 as verified by sequencing of the PCR product. The RACE PCR technique was used to obtain cDNA for the rest of the T*as*1r1 sequence from mRNA of pig taste buds. Candidate PCR products were sequenced and the RACE process was repeated until the full length mRNA sequence was obtained. Full length products were amplified by PCR using Platinum Taq DNA Polymerase High Fidelity (Invitrogen Co., 11304-011) and cloned into pCR8/GW/TOPO TA vector (Invitrogen K2500-20). Both sequences were then verified and the open reading frame was recombined into pcDNA6.2/V5-DEST Gateway vector (Invitrogen Co., 12489-027) using Gateway BP clonase II enzyme mix (Invitrogen Co, 11789-020) for expression in mammalian cells.

Materials for the stable cell reporter system construction

The stable cell reporter system of pT*as*1r3/pT*as*1r1 cell line was developed at the Celltec-UB research group in the Cell Biology Dept. from the University of Barcelona (Spain). All the materials used in its construction were as follows: The pT*as*1r3-pT*as*1r1-Gα15 NFAT-*bla* CHO-K1 cell culture using the Geneblazer® technology (Gα15-NFAT-*bla* CHO-K1 master cell line, Invitrogen Co., K1213). Gateway® cloning technology (Gateway® BP Clonase TM II mix enzyme -Invitrogen 11789020, LR Clonase TM II enzyme mix –Invitrogen Co., 11791020-) was used to obtain constructions in different vectors. Plasmids used for construction were pcDNA/V5/GW/D-TOPO, pvr8/GW/D-TOPO, pcDNA 6.2 Hygro/dest (Invitrogen Co., K1233), pEGFP-C1 (Elim biopharmaceuticals Inc. Id peGFP-C1), pcDNA 6.2/V5/GWD-TOPO pt1r1 and pcDNA 6.2/V5/GWD-TOPO pT*as*1r3 (Animal Science Dpt., University of California, Davis, CA, USA). The lipofection process was performed using Lipofectamine™2000 reagent (Invitrogen Co., 11668-027) and Opti-Mem 1x (Invitrogen Co., cat.nº31985-047). Transfected cells selection was carried out using Geneticin G418 sulphate (Invitrogen Co, 11811-023) and Hygromicin (Invitrogen Co., 10687), and for stable cells selection were used penicillin/streptomycin (P/S,1000mg/ml, Lonza, 17-602E), zeocin (100mg/ml, Invitrogen Co., R-250-01) and blasticidin S HCl (5mg/ml, Invitrogen Co., R210-01). The RNA extraction and RT-PCR reagents were the RNA PureLink™ Micro-to-Midi™ Total RNA Purification System (Invitrogen Co, 12183-018), reverse transcription reagents SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen Co, 11752-050) and PCR reagents Platinum taq DNA polymerase (Invitrogen Co, 10966034). Readings were carried out using the LiveBLAzer™-FRET B/G loading kit with CCF4-AM (Invitrogen Co., K1085) and SAFIRE automatic dual wave-length bottom-reading fluorescence plate reader (Tecan). Culture components for the stable pt1r1/pt1r3 cell line were DMEM GlutaMAX ™ (Gibco, 31966-047), 10% dialyzed Fetal Bovine Serum (FBS, Gibco, 26400), HEPES 1M (pH 7.3, Invitrogen Co., 15630-056) and MEM non-essential amino acids 100x (Invitrogen Co., 11140-035). Test β-lactamase assays required a different cell medium, 99.9% DMEM (4.5g/l glucose, LONZA, BE12-614F), BSA (Sigma-Aldrich, A9647) and PROBENECID (200mM, Sigma-Aldrich, P8761) for the application of the LiveBLAzer™-FRET B/G loading kit with CCF4-AM (Invitrogen Co., K1085) and just prior to reading, media was changed to 99.5% DMEM (4.5g/l glucose, LONZA, BE12-614F) and 0.5% of FBS (Gibco, 26400).

The pT*as*1r1 and pT*as*1r3 cloning and stable cell line construction

The cDNA from pT*as*1r3 and pT*as*1r1 sequences, subcloned into pcDNA 6.2/V5/GWD-TOPO expression vector (Invitrogen, Co.), were obtained from the University of California Davis (Davis, CA, USA) through two plasmids, pcDNA 6.2/V5/GWD-TOPO pT*as*1r1 and pcDNA 6.2/V5/GWD-TOPO pT*as*1r3. Their complete sequences were verified and subcloned in different eukaryotic vectors using the Gateway® cloning technology, obtaining constructions in the following vectors: pcDNA/V5/GW/D-TOPO, pvr8/GW/D-TOPO and pcDNA 6.2 Hygro/dest. All plasmids were fully sequenced after construction ("Servicio de Secuenciación de DNA del Instituto de Biología Molecular de Barcelona", Jordi Girona 18-26, 08034, Barcelona, Spain). To obtain stable cell expression lines, commercial CHO-K1 cells (GeneBlazer® Gα15-NFAT-*bla* CHO-K1 master cell line) were transfected by lipofection and their selection was applied after 48h using geneticin (700µg/ml) and hygromicin (800µg/ml) for at least 4 weeks. This commercial cell line contains β-lactamase as gene reporter that is controlled by the transcriptional nuclear factor of activated T cells (NFAT). The pEGFP-C1, plasmid DNA encoding green fluorescent protein, was used as a transfection control. Colonies were isolated, amplified and characterized by RT-PCR using the following primers for each gene: pT*as*1r1 forward 5'-GTAGCTGGCCTCTTCCCTCT-3', pT*as*1r1 reverse 5'-TCCGAGCACACGTCGTATAG-3', pT*as*1r3 forward 5'-AGGAAATCAACAACGGATCG-3' and pT*as*1r3 reverse 5'-CTGCGTGTAGTCGCAGTAGG-3'. The thermal cycling parameters for RT-PCR were 25ºC for 10 min, 50ºC for 30 min. and 85ºC for 5 min, and for the standard PCR were 95ºC for 5 min, followed by 35 cycles of 95ºC for 30s, 58ºC for 60s and 72ºC for 15s, and finally 72ºC for 5 min. Stable cells were generated using antibiotic selection with P/S (1000mg/ml), zeocin (100mg/ml) and blasticidin S HCl (5mg/ml) containing stable pT*as*1r1/pT*as*1r3 cell line growth medium. These stable selected lines were again amplified and verified by RT-PCR and finally maintained in liquid nitrogen.

Cell cultures

Cell lines were maintained between a density of $7.5x10^5$-$1.5x10^6$ cells and 90% of confluence at 37ºC and 5% CO_2 conditions. Cell lines used in our cell reporter experiments were as follows: the stable transfected cell line pT*as*1r1- pT*as*1r3-Gα15-NFAT-*bla* CHO-K1 and the non-transfected cell line Gα15-NFAT-*bla* CHO-K1, used as a negative control, with P/S (1000mg/ml, Lonza, 17-602E), zeocin (100mg/ml, Invitrogen Co, R-250-01) and blasticidin S HCl (5mg/ml, Invitrogen Co., R210-01) containing growth medium- DMEM GlutaMAX ™ (Gibco, 31966-047), 10% dialyzed Fetal Bovine Serum (FBS, Gibco, 26400), HEPES 1M (pH 7.3, Invitrogen Co., 15630-056) and MEM non-essential amino acids 100x (Invitrogen

Co., 11140-035)-, and the CMV-*bla* CHO-K1, which is a cell line designed to express β-lactamase reporter gene by control of cytomegalovirus promoter, used as a positive control, with only P/S (1000mg/ml) and the same growth medium as transfected and non-transfected cells.

Assay design

Functional assays were performed by using glutamine 100mM as inducer. Briefly, cells (pT1r1- pT1r3-Gα15 NFAT-*bla* CHO-K1) were exposed to inducer for 24h or 48h after seeding at a cell density of 20.000 cells/well in 96-well plates (NUNC). Positive control of the assay (CMV-*bla* CHO-K1, –CMV-), negative control of the assay (Gα15-NFAT-*bla* CHO-K1,-NFAT-), negative control of the induction (pT1r1-pT1r3-Gα15-NFAT-*bla* CHO-K1, -NFAT1+3-) and positive control of the induction (pT1r1-pT1r13-Gα15 -NFAT-*bla* CHO-K1 + Glutamine 100mM, -NFAT- 1+3+Gln-) were plated in the first row of each plate (triplicates), the rest of wells had the NFAT1+3 cell line to test the amino acid experimental treatments (triplicates/treatment) (*Figure 1*).

After 48h incubation (37ºC, 5%CO_2), cell media components were changed, adding 100μl/well of media without glutamine and only 0.5% of FBS (0.5% media). Glutamine starved cells were incubated overnight (37ºC, 5% CO_2). On the following day, test and positive induction control wells were prepared in advance in transition plates. In test wells, Gly and L-amino acids and their different concentrations were diluted with 0.5% culture medium (total volume 120μl/well and triplicates). Next, culture medium from experimental plates was removed and 100μl/well of every transition plate well dilution was added. After addition of test compounds and controls, plates were incubated (37ºC, 5%CO_2) for 5h. At the end of the incubation period, culture medium was removed from experimental plates and the specific β-lactamase assay medium was added, keeping them at room temperature.

Figure 1 > Plate design including in the first row all the controls (triplicates). The rest of rows had the pT*as*1r1-pT*as*1r13-G?15-NFAT-*bla* CHO-K1 cell line with different amino acid solutions (triplicates).
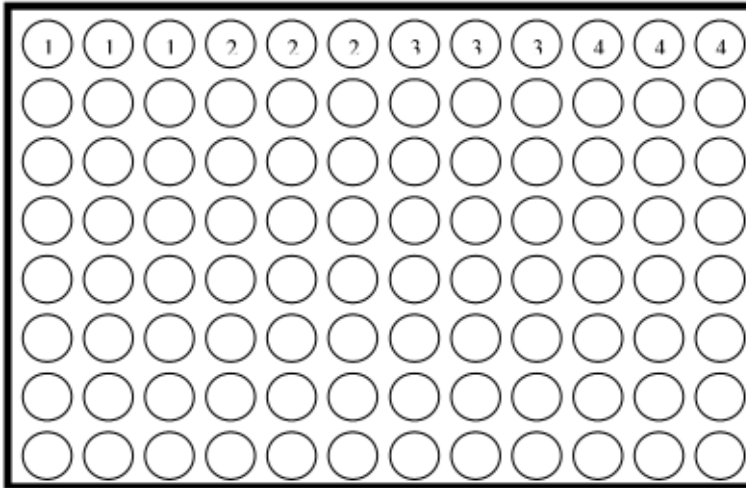
1 - Positive control of assay: CMV-*bla* CHO-K1 cells.
2 - Negative control of assay: non-tranfected cells or NFAT.
3 - Negative control of induction: transfected cells without L-Glutamine.
4 - Positive control of induction: tranfected cells with L-Glutamine.

Six CCF4-AM substrate loading solution was prepared following manufacturer's directions (LiveBLAzer™-FRET B/G loading kit with CCF4-AM, Invitrogen Co., K1085) and it was added to experimental plates (20µl/well). These plates were incubated at room temperature for 2h. Finally, culture media with CCF4-AM substrate was removed followed by two consecutive washes of PBS 1x (100µl/well). Induced β-lactamase activity was determined by fluorescence measurement using SAFIRE automatic dual wave-length bottom-reading fluorescence plate reader (Tecan) with excitation wavelength 402/20 nm and emission scans at 460 nm (blue) and 530 nm (green).

Receptor stimulation would elicit a final increase of intracellular Ca^{+2} levels that only lasts a few seconds (transient) although it is turned in a long response through the NFAT and β-lactamase gene reporter construction (stable). This signal of calcium leads to a conformational change and traslocation from the citosol to the nucleus of NFAT. The NFAT joins to a promoter that controls the expression of β-lactamase gene reporter and triggers its expression. The expression of this gene reporter is detected by fluorescence resonance energy transfer (FRET) through the CCF4-AM fluorochrome that is hydrolyzed by β-lactamase. If the CCF4-AM remains intact, the

excitation at 410 nm (between 402-420nm wavelengths) is emitted at 530nm (green) whereas if the CCF4-AM is fragmented by β-lactamase, it is emitted at 460nm (blue).

Figure 2 > Fluorescence microscopy imaging of transfected and non- transfected Gα15-NFAT-*bla*CHO-K1 and CMV-*bla* CHO-K1 cell lines (Adapted from Invitrogen ®).

No response	Good Response	Constitutively Active

Negative control
Negative control of induction
No response transfected cells

Positive control of induction
Good response tranfected cells

CMV-*bla* CHO-K1
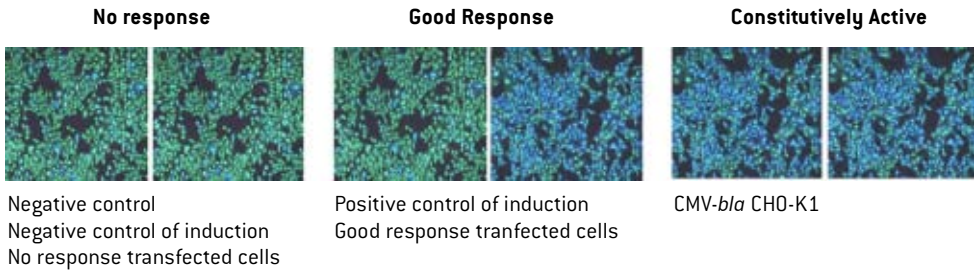
These recordings were used to calculate the blue/green ratio (B/G), which quantifies the ligand activity in stimulating the umami receptor. The B/G ratio was obtained by dividing subtracted 460 nm reading (blue) by the subtracted 530 nm value (green) for every well.

Tested L-amino acids and concentrations

All experimental amino acids were supplied by Sigma-Aldrich® (Tres Cantos, Madrid, Spain), and were cell culture grade. L-Alanine (L-Ala), L-Arginine (L-Arg), L-Asparragine (L-Asn), L-Cys (L-Cysteine), L-Gln (L-Glutamine), Monosodium glutamate (MSG), L-Glu (L-glutamic acid), Glycine (Gly), L-Histidine (L-His), L-Isoleucine (L-Iso), L-Leucine (L-Leu), L-Lysine (L-Lys), D,L-Methionine (D,L-Met), L-Phenilalanine (L-Phe), L-Proline (L-Pro), L-Serine (L-Ser), L-Threonine (L-Thr), L-Tryptophan (L-Trp) and L-Valine (L-Val) were tested at 50, 5 and 0.5mM. L-Aspartame (L-Asp) was tested at 33, 5 and 0.5mM. Finally L- Cystine (L-Cis) and L-Tyrosine (L-Tyr) were assayed at 4.6 and 0.5mM and at 2.50 and 0.50mM, respectively. Different concentrations from L-Asp, L-Cis and L-Tyr were due to low solubility at 50 and 5mM. All amino acids and concentrations were tested in triplicates in five different assays. The fluorescence background used to calculate the stimulation of the porcine umami taste receptor in test wells was the mean of B/G ratio from the control NFAT1[+3] in every assay. Therefore, the final value of taste receptor stimulation or response ratio (RR) for each amino acid tested was the ratio between the B/G values of each test well and the corresponding calculated background (the average value from the NFAT1[+3] triplets well).

Amino acid *in vivo* data

Data of preferences for some essential amino (L-Lys, D,L-Met, L-Trp and L-Thr) and non-essential amino acids (L-Glu, L-Gln, Gly, L-Ala and MSG) at 0.5, 5 and 50mM from Chapter 1 were used to compare with the *in vitro* results.

Statistical Analysis

The RR was used for statistical analysis. The RR from triplicates that were 2 times higher than the SD of them was considered a missing value. To obtain the RR normality, these variables were transformed to 1+Log10. The RR of Gly and L-amino acid and their concentrations were analysed using the MIXED procedure in SAS, with assay and well treated as random effects according to the following model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the dependent variable for the observation; μ is the general mean for all observations; α_i is the fixed effect of amino acid; β_j is the fixed effect of concentration; $(\alpha\beta)_{ij}$ is the interaction between amino acid and concentration; and ε_{ij} is the residual error.

P-values were adjusted using the Tukey correction where differences among amino acids and concentrations were considered significant when $P < 0.05$.

The average of RR was compared to the neutral value of 1 (NFAT1+3) using a Student's t test.

Pearson's correlation coefficients and regression analysis between RR and percentage of preference of pig preferred amino acid solutions (Gly, L-Glu, L-Gln, MSG and L-Ala) at 0.5, 5 and 50mM (*Chapter 1*) were obtained using the CORR and STEPWISE procedures in SAS, respectively.

All the statistical analyses were carried out with the statistical package SAS® (version 9.1., Software SAS Institute Inc., Cary NC, 1991-2001).

Results and discussion

The heterodimeric porcine umami taste receptor (pT1r1 and pT1r3) has been identified in pig circumvallate papilla tissue samples (*Table 1*). Both the pT*as*1r1 and the pT*as*1r3 gene sequences begin with a methionine codon which is required for all mammalian protein synthesis. They translate into a pT1r1 receptor containing 844 amino acid residues and a pT1r3 receptor with 855 residues.

Table 1 > Pig T*as*1r1 (2535bp) and T*as*1r3 (2568bp) gene sequences.

GENE	SEQUENCE
pT*as*1r1	ATGTTGCTCTGGGAGGCGTGCCTGCTGGGTCTGCAGCTCTCCCTCTCCTGCTGCTGGGCTTTGGGCTGCCAGGGCCCAGAGTCCTCCCCTGACTTCAGCCTCCCTGGGGACTACCTTGTAGCTGGCCTCTTCCCTCTACACGGTGACCACCCAGAGGCGAGGCGCAGACCCACAGTGGCCTTCTGTGACAGACCCAACACCTTCAACGGCCACGGCTACCACCTCTTCCAAGCCATGCGGTTTGGCATCGAGGAGATAAACAACTCCAGCGCCCTGCTCCCCAACGTCACTCTGGGGTATGAGCTATACGACGTGTGCTCGGAGTCCGCGAACGTGTACGCCACGCTGAGCATCCTGAGCAAGGCTGGGACAGGCTACGTGGACATCCAAGAAAACCCTGCCCTCTATTCCCCCAAGACCGTGGCGGTCATCGGGCCCGACACCACCAGCCGCGCTGCCACCACCGCAGGCCTGCTGGGCCCCTTCCTGGTGCCCCTGGTCAGCTATGATGCCAGCAGCACGGTGCTGGGTGTGAAGCGGGTGTACCCCTCTTTCCTGCGCACCATCCCCAGCGATGACCACCAGGTGGAGGTCTTGGTGCTGCTGCTGCGGAGGTTCAGGTGGGACTGGATCTCGGTGGTGGGCAGCGAAGGTGACTACGGGCAGCTGGGGGTGCAGGCGCTGGAGGACAGGCCCGCCGGCAGGGCATCTGCATTGCCTTCAAGGACATCGTGCCGTTCTCTGCCCAGCCGGGCGACCAGAGGATGCAGAGCATGATGGGCAGCCCGGCTCTCGCAAGGACCACCGTTGTGGTCGTTTTCTCCGGTCGGCAGCTGGCCAGGGTGTTCTTTGAGTCTGTGGTGCTGGCCAACTGACCTCCAAGGTGTGGGTCGCCTCCGAGGACTGGGCCATCTCCAGACACATCAGCAGTGTGCCCGGGATCTCGGGGCATCGGCACAGTGCTGGGCGTGGCCATCCCCGGGAGGCTGGTGCCCGGCCTGAAGGAGTTTGAGGAGGCCTACGTCAGGGCAGAGAAAGGGGCCCTTGGGCCTTGCCCCAGGGGCTCCTGGTGCAGCACCAACCAGCTCTGCACAGACTGCTGGGCTTTCACGGCCCAGGAGATGCCCACACTTGGAGCATTCTCCATGAGTTCTGCCTACAACGTGTACCAGGCTGTCTATGCCGTGGCCCAGGGCCTCCACCAGCTCCTGGGCGTGTGCCTCTGGCATCTGTTCTCGAGGCCAAGTCTACCCCTGGCAGCTCCTGGAGGAGATCCGCAAGGTCAATTTCCTTCTACGCAAGCACACTGTGACATTTAGTGACAGTGGGGACTCGCTCAGCGGCTATGACATAATTGCCTGGGACTGGAGTGGCCCCAAGTGGACCTTCAGGGTCATCGGCTCGTCATCGTCGTCATTGTTGTCGCCAGTTCAGCTAGACATAAATACTACCAAAATCCCATGGCGTGGCGGGGACAACCAGGTGCCGACGTCCGTGTGTTCCAGCGACTGTCCTGAGGGCCACCAGAAAGTGATCATGAGTTTCCACCACTGCTGCTTTGAGTGTGTGCCTTGTGAGGCCGGGACCTTCCTCAACGAGAGTGACCCCTACAGCTGCCAACCCTGTGGGAAAGAAGAGTGGGCCACCTGAGAGAAGCCAGACTTGCTTCCCACGCACCGTGGTATTCTTGACTTGGCATGAGCCCATCTCTTGGGTGCTGCTAGCAGCTAACACGATGTTGCTGCTGCTGGTGGGCTGGGACTGCTGGCCTGTTTCTTGGTTGCGCTTGGTGGTGGCCACCATGCTTGCTGCGCCAAGGCCTCTTTGCCCTTGGTTTTGCCATCTTCCTGTCCTGCCTGACAATCCGCTCTTTCCAACTTGTCTTCATCTTCAAGTTTTCTGCCAAGATGCCCACATTCTTTCACGCGTGGGTCCAAAACCATGGTGCTGGCCTGTTTGTTGGTGATCAGCCTCGATAACCCAGCTGCTTATCTGTCGGTTTGGCTGGCGGTGTGGACCCCACTGCCCACCAGGGAATACCAGCGCTTCCCACAGCTGGTGGTGCTTGACTGCACAGAAGCGAATTCACTGGGCTTAAGGCTGGCTTTCACCTACAACTTCGTGTCCTGGATCACCTTCTTCACCGTGGCCAGTGTCTACAGGGGCAAGTACCTGGCCGCCGTCAACGTGTTTGCCATGCTGAGCAGCCTGAGTGGAGGCTTCAGCGGTTATTTCCTCCCCAAATGCTATATCATCCTGTGCCGCCCCGATCTCAACAGCACGGAGCACTTCCAGGCCTCCATCCAGGACTACAGCAGGCGCTGCGGCTCCACCTGA
pT*as*1r3	ATGCCAGGCCTGACTCTCCTGGGCCTCACAGCTCTCCTGGGCCTCGGGGCGGGGGCCCCGCTGTGCCTGTCCCGGCAGCTCAGCATGCAAGGGCACTACGTGCTGGGGGGGCTCTTCCCCCTGGGCTCCACCGAGGATGCTGGGCTGGGCGACAGGACAGAGCCCAACGCCACCGTGTGTACCAGGTTCTCGTCCCTCGGCCTGCTCTGGGCACTGGCCATGATGATGGCTGTGTGGAGGAAATCAACAACGGATCGGCCCTGCTCCCTGGGCTGCGCCTGGGCTACGACCTCTTCGACACCTGCTCAGAGCCCATGGTCGCCATGAAGCCCAGCCTAGTGTTCATGGCCAAGGCTGGCAGCCGCATTGCCACCTACTGCGACTACACGCAGTACCAGCCCCGCGTGCTGGCCGTCATCGGGCCCCACATGTCTGAGGTCGCCCTGGTCACCGGCAAGTTCTTCGGCTTCTTCCTCATGCCGCAGGTCAGCTACAGCGCCAGCACGGACCGGCTGAGCAACCGCGAGACGTTCCCCTCCTTCTTCCGCACGGTGCCCAGCGACCGCGTGCAGGCAGTGGCCATGGTGGAGCTGCTGCAGGAGCTGGGTTGGAACTGGGTGGCCGCCGTGGGCAGCGACGACGAGTACGGGCCGGCAGGGCTGAGCCTCTTCTCAGCCTTGGCCGCAGCTGGGAGTATCTGCATCGCGCACGAGGGCCTGGTGCCGCTGCCCCACGCCCGTAGCCCAAGGCTGGACTGCACGCAGGGCCTGCTGCCCCAAGTGAACCAGAGCAGCGTGCAGGTAGTGGTGCTGTTCTCCTCCGCCCGCGCCGCCCGAGCTCTTCAGCCACAGCCTCCACTGCAGGCTCTCGCCCAAGGTGTGGGTGGCCAGCGAGGCCTGGCTGACCTCGGACCTGGTCATGACACTGCCCGGGCATGGACCAGGTGGGCACCGTGCTCGGGTTCCTGCAGAGCGGTGGCCTCGGGGATGGGGCCTCGGTTCCTGCTGACGGCAGGGCCTGGAGGAGCACGTGCTGGGGGCCTGCTGCCCCCAGTGTGACTACGTCACATTGGAGAGCCTGTCCGAGGGGCTGCTGCACCACCGGAGCTTCCCCGCCTACGCGGCTGTGTACAGCGTGGCCCAGGCGCTGCACAACACGCTGCTCTGCAACGCCTCGGGCTGCCCCACGCGGGGAGCCGGTGCTGGCTGCCCTGAGCCGGGCGGCAGGGGCTGGAGGAGCACGTGCTGGGGGCCTGCTGCCCCCAGTGTGACTACGTCACATTGGAGAGCCTGTCCGAGGGGCTGCTGCACCACCGGAGCTTCCCCGCCTACGCGGCTGTGTACAGCGTGGCCCAGGCGCTGCACAACACGCTGCTCTGCAACGCCTCGGGCTGCCCCACGCGGGGAGCCGGTGCTGGCTGCCCTGAGCCGGGCGGCAGGGGCTGGAGGAGCACGTGCTGGGGGCCTGCTGCCCCCAGTGTGACTACGTCACATTGGAGAGCCTGTCCGAGGGGCTGCTGCACCACCGGAGCTTCCCCGCCTACGCGGCTGTGTACAGCGTGGCCCAGGCGCTGCACAACACGCTGCTCTGCAACGCCTCGGGCTGCCCCAC

Similar to other mammalian species, our current findings predict that the pig umami taste receptor is located in the plasma membrane and their sequence contains an extracellular N terminus ligand binding domain from amino acid 1 to 571 in pT1r1 and 1 to 565 in pT1r3, 7 transmembrane domains and an intracellular C-terminal tail, commonly found in GPCR structures (Siehler, 2008). Amino acid homologies with predicted pig umami proteins were lower than nucleotide homologies for all the animals studied. Among the other mammals evaluated, the porcine nucleotide and amino acid sequences were found to have the highest homology to the cat umami receptor and the lowest to mouse and humans (*Table 2*). According to our characterization, human and mouse umami perception may not be a good model for pig umami sensing since differences in T1R sequences within and between species can influence the selectivity and specificity of taste responses (Nelson et al., 2002; Reed et al., 2004; Manita et al., 2006; Raliou et al., 2009).

Table 2 > Nucleotide and amino acid homologies of the predicted pig umami heterodimer taste receptor genes and proteins (pT*as*1r1/pT*as*1r3 and pT1r1/pT1r3, respectively) compared to several mammalian species.

Animal Species	Nucleotide		Protein	
	pT*as*1r1	pT*as*1r3	pT1r1	pT1r3
Human	81%	83%	75%	74%
Cat	85%	86%	81%	81%
Dog	82%	85%	81%	83%
Cow	85%	84%	80%	77%
Mouse	79%	74%	71%	72%
Horse	84%	ND	77%	ND

The pT*as*1r1 and pT*as*1r3 gene sequences (*Table 1*) were used to construct the stable cell reporter system that co-expresses both genes with a promiscuous Gα15 protein linked to the parental cell line NFAT-*bla* CHO-K1. The cell line integrates β-lactamase as gene reporter and the transcriptional factor that regulates its expression known as the nuclear factor of activated T cells (NFAT). This *in vitro* system was used as a screening tool to quantify responses to potential pig umami ligands, Gly and L-amino acids.

In vitro responses to amino acids were a function of the nature of the amino acid in solution (P<0.01), its concentration (P<0.02) and the interaction of the amino acid type by concentration (P<0.01). The L-Ser, aromatic amino acids (L-Tyr and L-Phe), sulphur amino acids (D,L-Met, L-Cys and L-Cis) and the majority of the essential amino acids (EAA) (L-Lys, L-Trp, L-Iso, L-Leu and L-Val) did not elicit any response at any concentration.

In contrast, several non-essential amino acids (NEAA) stimulated the cell system. The strongest responses (P<0.05) were observed with L-Glu, MSG (both are umami tastants in mice and humans), L-Gln, L-Ala and L-Asn in all concentrations (0.5, 5 and 50mM), showing the highest response at 50mM. The Gly also stimulated the receptor although the response was lower (P<0.05) than the previous L-AA.

Other NEAA such as L-Pro and L-Asp only stimulate the receptor at one of the concentrations tested, similar to the EAA L-Thr and L-Arg. The L-Arg is considered an EAA in young piglets (NRC, 1998). These results showed that the porcine umami taste receptor is mainly stimulated by NEAA (L-Glu, MSG, L-Gln, L-Asn, L-Ala and Gly) and can be referred to as umami tastants in pigs (*Figure 3*). The NEAA are present at high levels in protein-rich ingredients and piglet feeds, where they are made by the body from the EAA or by the normal breakdown of ingested proteins. Therefore, the umami taste receptor in pigs is detecting an essential macronutrient, protein.

Figure 3 > Porcine umami cell reporter system responses to Gly and L-amino acids solutions at 0.5, 5, and 50mM. Each bar represents the Mean±SEM of each amino acid response ratio (RR). Amino acid responses compared to the neutral value (NFAT 1+3=1).

Tested amino acids at 0.5mM
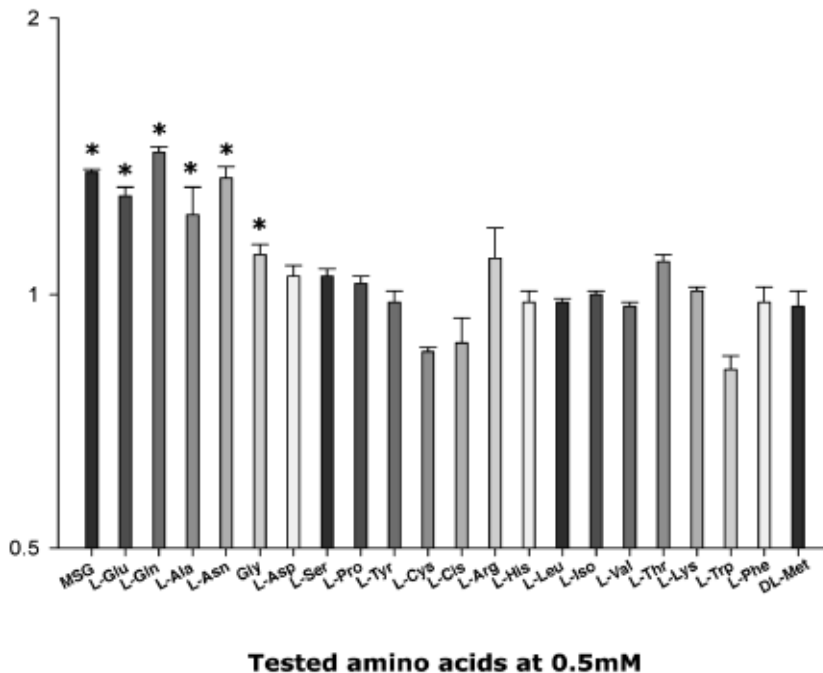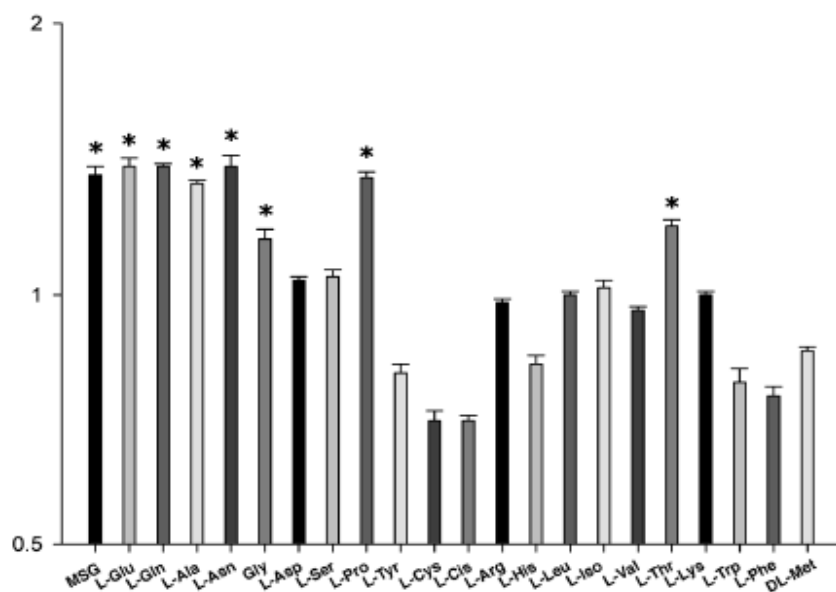
Tested amino acids at 5mM
except L-Cis (4.6mM) and L-Tyr (2.5mM)
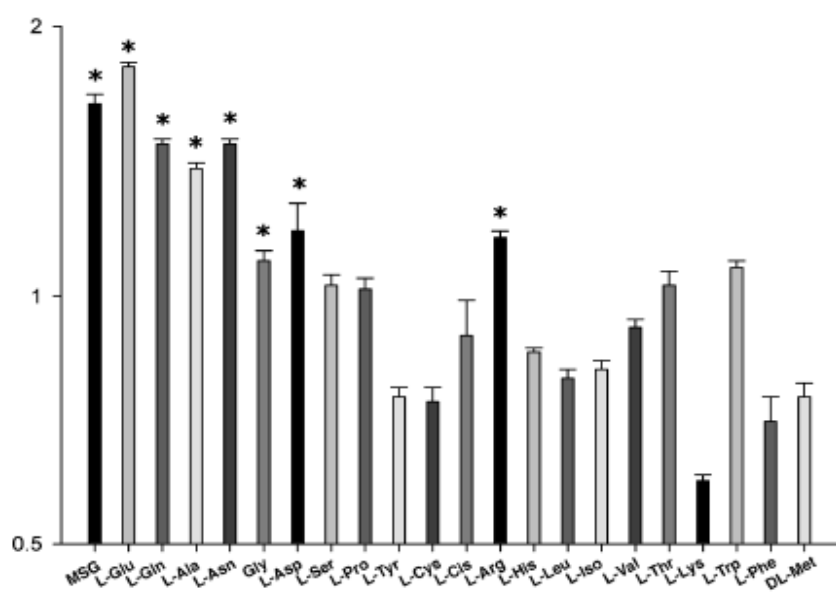
Tested amino acids at 50mM
except L-Asp (33mM), L-Cis (4.6mM) and L-Tyr (2.50mM)

Mice *in vitro* trials show that the T1R3 and T1R1 dimer functions as a broadly tuned sensor responding to most of the 20 standard L-amino acids but not responding to their D-enantiomers. In humans the receptor preferentially responded to L-Glu and L-Asp, and this response synergised with IMP and GMP (Yamaguchi and Ninomiya, 2000). On the other hand, some L-amino acids that had been described as sweet in humans such as L-Ala, L-Gln, L-Thr, L-Arg, L-Pro and Gly stimulated the mouse umami taste receptor (Nelson et al., 2002, Matsunami et al., 2000; Li et al., 2002; Nelson et al., 2002; Sako et al., 2003; Zhao et al., 2003). Zhao et al. (2003) tested behavioural responses of T1R1 and T1R3 knockout mice to L-amino acids, and observed a complete loss in preference for umami tastants in both strains. These *in vivo* trials confirmed the *in vitro* results from Nelson et al. (2002). Our pig model *in vitro* data only showed modest similarities with those obtained from human (L-Glu, MSG) and mice (L-Glu, L-Gln, L-Ala, Gly). This divergence may be related to differences in T1R1/T1R3 sequences of the pig compared to human and mice (*Table 2*).

The *Figure 4* shows the *in vitro* amino acid responses as the mean of all tested concentrations. The MSG, L-Glu, L-Gln, L-Asn and L-Ala resulted in higher responses than the rest of amino acids (P<0.01). These differences may suggest that there are multiple mechanisms involved in detecting and signalling amino acids and umami stimuli in pigs.

The heterodimers of umami and sweet taste receptors share a common unit, the T1R3. Zhao et al. (2003) carried out behavioural assays to test high concentrations (300mM) of "sweet" L-amino acids (L-Ala, L-Thr and L-Ser), showing that T1r1 knockout mice, but not T1r3, retained a small residual attraction, likely reflecting some activation of the sweet taste receptor. Our results showed that the pig umami taste receptor was stimulated by several L-AA considered sweet in humans, some highly stimulated the pig umami taste receptor (L-Gln, L-Ala, Gly) and others that triggered a response depending on their concentration (L-Pro, L-Asp, L-Thr, L-Arg) (*Figure 3*).

Figure 4 > The porcine umami cell reporter system responses to Gly and L-amino acid solutions (values represent the mean of all tested concentrations). Each horizontal bar represents the Mean±SEM of each amino acid response ratio (RR).
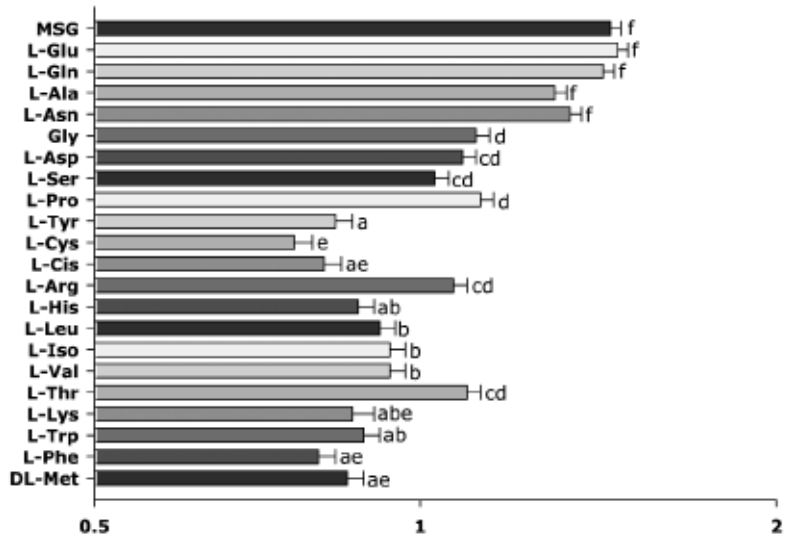
Different letters mean significant differences among amino acid solutions at P<0.01.

Our pig *in vitro* and *in vivo* results showed that L-Glu and MSG had similar responses at all concentrations (Chapter 1), although in human sensorial tests they are perceived as two different profiles in non-buffered solutions, sour and meaty-salty, respectively (Pérez-Portabella et al., unpublished data). Both were highly preferred by pigs and highly stimulated the umami taste receptor. The L-Ala and L-Gln, considered as sweet tastants in humans, were also strong umami ligands, in the meaning of they were highly stimulating the pig umami receptor, and were preferred by pigs. The Gly may be also considered as umami tastant in pigs although its *in vitro* response was lower than the rest of the umami L-AA (Figure 4). Behavioural studies in rats using the conditioned taste aversion to MSG and discrimination experiments with taste of sodium ion as the control showed that MSG aversion was generalized to L-Ala, Gly and L-Ser and moderately to L-Arg, and that rats were not able to discriminate between MSG and L-Asp, Gly, L-Pro and L-Ser, indicating that these amino acids were perceived very similar to MSG (Delay et al., 2007). Differences in taste perception of umami ligands may open the possibility that each amino acid has different binding affinities or different binding sites within the same taste receptor, or that there might be multiple afferent pathways for signalling L-AA based on umami sensing (Delay et al., 2007).

An alternative way to evaluate a gustatory stimulation involves making electrophysiological recordings on the whole nerve (Chorda tympani –CT- and Glossopharyngeal –GP- nerves) or in single fiber responses during taste stimulation. Hellekant and Danilova (1999) defined different taste clusters in CT and GP nerves in pigs. The MSG alone or enhanced with IMP and GMP elicited higher responses in CT than GP, in the mineral (M) and sweet (S) clusters. The Gly also elicited higher responses in CT than GP, in the S cluster, although some activity was also observed in bitter (Q) and sour (H) clusters, which may deter from its attractiveness because stimulation of these clusters elicits rejection to the compound (Danilova et al., 1998a, 1998b). Danilova et al. (1999) also reported another study on stimulation of CT and GP in piglets, and both nerves exhibited again large responses to MSG and Gly. They also showed that responses to MSG were not attributed only to sodium ions, since NaCl gave a smaller response in CT nerve. In our *in vivo* studies (Chapter 1), sucrose (positive control) resulted in the same preference than MSG and L-Glu. The MSG and L-Glu were highly preferred and pigs showed a high appetence to consume them. In our trials Gly also showed similar responses to sucrose except at the highest concentration (500mM) that may be explained by the stimulation of the bitter afferent fibers (Q cluster) that may mediate a rejection response to the test solution. Thus, both our behavioural (Chapter 1) and *in vitro* data confirms and further explains some of the published electrophysiological porcine CT and GP recordings from Danilova et al. (1998a and 1998b).

The amino acids of our *in vivo* tests may be divided into two categories: 1) NEAA highly present on piglet diets: Gly, L-Glu, L-Gln, MSG and L-Ala, and 2) EAA whose dietary inclusion is commonly required to balance piglet feeds: D,L-Met, L-Lys ,L-Thr and L-Trp. Correlations and regressions between *in vivo* and *in vitro* data were only performed in the NEAA that resulted in significant responses of the porcine umami cell system. The EAA did not stimulate the receptor at any tested concentration (Figure 3). At 50mM *in vitro* results were positively correlated with pig preferences for all NEAA (R= 0.8487, P= 0.06) and more specifically for L-Ala, L-Gln, L-Glu and MSG (R=0.9173, P<0.05) which resulted in the highest responses in both (*in vitro* and *in vivo*). A regression analysis showed a linear relationship between the cell reporter system and behavioural responses for Gly, L-Glu, L-Gln, MSG and L-Ala at 50mM, with a R2=0.7202 (P=0.06, CV=2.78) (Figure 5).

Figure 5 > Linear regression between the porcine umami cell reporter system response ratios (RR) and the percentage of preference to NEAA Gly, L-Ala, L-Glu, L-Gln and MSG at 50mM.
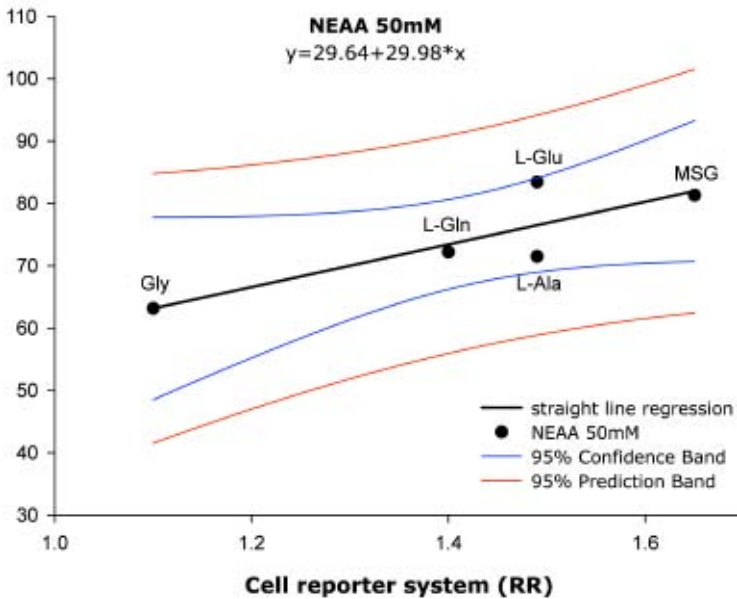
Looking at these results, our *in vitro* cell reporter system proves to be an appropriate prediction tool to identify umami ligands highly preferred by pigs. On the other hand, further research looking at this synergism with IMP and GMP need to be conducted to determine if pT*as*1r3/pT*as*1r1 behaves like in other mammals where amino acids with lower responses behave as umami ligands in the presence of nucleotides.

The umami taste stimulus may indicate the presence of protein (Boughter and Bachmanov, 2007) and a pleasant taste (Bellisle, 1999). Therefore, our *in vitro* system may recognize sources of protein adequate to stimulate the pig voluntary feed intake, a critical issue in swine production particularly in the weaning process.

Conclusions

The heterodimeric porcine umami taste receptor has been identified and characterized consisting of two sequences of 844 (pT1r1) and 855 (pT1r3) amino acid residues. Similar to the umami receptor in other mammalian species, the porcine receptor responds to a classic GPCR structure with one predicted extracellular ligand binding domain from amino acid 1 to 571 in pT1r1 and 1 to 565 in pT1r3 and seven predicted transmembrane domains. Pig T1r1/T1r3 heterodimer has been found to share the highest homologies with the cat umami receptor and the lowest with the mouse and the human. A stable cell reporter system expressing the pT*as*1r3/pT*as*1r1 genes was constructed to identify umami tastants and quantify their responses in pigs. The cell system showed significant responses to non-essential amino acids, L-Glu, MSG, L-Gln, L-Asn, L-Ala and Gly, what suggests that this receptor may be tuned to detect dietary protein sources. In contrast, essential amino acids L-Ser, L-Tyr, L-Phe, D,L-Met, L-Cys, L-Cis, L-Lys, L-Trp, L-Iso, L-Leu and L-Val did not elicit any response. The *in vivo* data was significantly correlated to the *in vitro* results meaning that the L-amino acids stimulating our cell reporter system were highly preferred by pigs. Therefore, we propose the use of the *in vitro* cell system as a tool to identify umami ligands that may be pleasant compounds to pigs. These umami tastants could then be used to develop new feeding strategies to increase the voluntary feed intake in critical stages of pig production, such as at weaning.

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Chapter 3

Age and dietary protein dependent porcine umami taste receptor gene (PT*as*1r1/Pt*as*1r3) expression in taste and non-taste tissue

Chapter 3 >>>>>>>>

Abstract

We investigated the expression of the porcine umami taste receptor genes, pT*as*1r1 and pT*as*1r3, in different tissues of the tongue's fungiform and circumvallate papillae, stomach, small intestine (duodenum, jejunum and ileum) and liver of pigs at birth, 26d old (preweaning), 48h after weaning (28d old) and 20d postweaning (46d old). Four pigs per age group were used. The effect of dietary crude protein (CP) content and amino acid supplementation of the diet from weaning to 20d postweaning (prestarter stage) on the expression of both genes was also assessed using three experimental diets: a high crude protein (HCP) diet containing 26% CP and 0.3% of supplemental DL-Methionine, and two 17% CP diets with (SAA) or without (LCP) supplemental L-Lys, D,L-Met, L-Thr, L-Trp, L-Val, L-Ile and L-His. Four pigs per diet were used. Real-time PCR was performed for relative quantification of changes in gene expression. Piglet performance parameters during the prestarter stage were also evaluated in 16 animals per diet. All age and dietary groups had half males and half females. Pigs from the LCP and SAA groups grew less than those from the HCP group (P<0.05). The pT*as*1r3 and pT*as*1r1 showed similar levels of expression regardless of age and dietary protein content, although overall pT*as*1r3 was more expressed than pT*as*1r1 in stomach and jejunum (P<0.05). Results of gene expression showed differences in pT*as*1r1 (P=0.06) and pT*as*1r3 (P=0.02) among tissues. Both genes were more expressed in tongue and stomach, followed by small intestine and liver. Changes in gene expression were observed with age, sex and dietary crude protein content (P<0.05). The main changes occurred after weaning, with more expression in males than females and in the LCP group that in the other two groups. We concluded that umami tastants are firstly detected in the tongue and their identification may continue along the GI tract through the porcine umami taste receptor. This chemosensory system in the pig is fully developed at birth and its expression may change with age (weaning), sex and dietary protein content. These changes may reflect the nutritional status of the pig and may help to develop new strategies using potential umami tastants to stimulate the voluntary feed intake in pigs.

Introduction

The development of novel strategic actions aimed to increase the voluntary feed intake during the most critical periods in pig rearing, such as weaning, have been a major focus in swine nutrition. Sensory perception of taste, odor, and texture influences food preference and consumption (Amarantos et al., 2001; Drewnowski et al., 2001), taste being a major determinant of food choice and voluntary intake (Wardwell et al., 2009). Elucidation of the mechanisms that help the animals to recognize the nutritional quality of feed and feedstuffs would help the development of dietary strategies that stimulate appetite for appropriate ingredients and lead to rejection of harmful substances.

The umami taste is considered as a pleasant taste (Bellisle, 1999) indicative of the protein fraction of the food (Ninomiya, 1998). The umami tastants elicit physiological responses mediated by afferent nerve fibers such as salivary flow stimulation, secretion of gastric juices and pancreatic cephalic phase stimulation (Bezençon et al., 2007; Kondoh and Torii, 2008), and they also activate several brain regions that play an important role in ingestive behaviour and regulation of food intake (Touzani and Sclafani, 2005; Tsurugizawa et al., 2008; Uematsu et al., 2009). Humans and mice perceive the umami taste via a trans-membrane receptor consisting of two G-protein coupled receptors (GPCR) referred to as T1R1 and T1R3 (Nelson et al., 2002; Li et al., 2002; Zhao et al., 2003). Similar to other mammals, the pig has the genes encoding for the umami taste receptor heterodimer pT1r1/pT1r3 (Chapter 2). Our group determined the sequence of the pT*as*1r1 and pT*as*1r3 genes extracted from circumvallate papillae. Compared to other known mammalian sequences, porcine genes showed the highest nucleotide and amino acid homologies with the cat and the dog and the lowest with rodents and humans (Chapter 2). Genetic variation in taste receptors may account for differences in food choices and dietary habits (García- Bailo et al., 2008). Therefore, mouse and human may not be a good model for the pig umami sensing.

Apart from the oral cavity (tongue and soft palate), T1R's are expressed in several non-taste tissues, where they have been considered as part of a diffuse chemosensory system (DCS). The DCS has been defined as the network of solitary chemosensing cells (SCC) found in epithelia of endodermal origin (i.e. respiratory and digestive). All SCC, such as the taste receptor cells (TRC), share the same signal transduction components (Sbarbati et al., 2009). Bezençon et al. (2007) determined in stomach, small intestine and colon of humans and mice that a chemosensory system exists based on the expression of taste receptors (T1R1, T1R2 and T1R3) and co-expression of

other cellular proteins needed for taste signal transduction such as $G\alpha_{,gust}$, PLC β-2 and TRPM5. The finding of SCC in the duodenal villous suggests an involvement of the DCS in nutrient sensing and absorption along the gastrointestinal tract (Bezençon at al., 2007; Mace et al., 2007; Jang et al., 2007 and Margolskee et al., 2007). Kiuchi et al. (2006) studied the genomic structure of the swine pT*as*1r3 and its expression in different tissues showing high levels in tongue papillae (fungiform and circumvallate), lymphocytes from the submucosal tissues of the lingual tonsil, kidney and testis (espermatogenic cells, mucosal epithelium and follicular B lymphocytes).

Taste responses are influenced by a range of genetic, physiological, and metabolic variables, and the impact of taste factors on food intake depends on sex, age and diet (Drewnowski, 1997; Tordoff, 2007). There are evidences of age-related changes in taste perception showing a decrease in taste sensitivity with age (Stevens and Cain, 1993; Mojet et al., 2003; Simchen et al., 2006; Wardwell et al., 2009). Also, females have normally been found to be less sensitive to NaCl and sucrose solutions than males but more sensitive to fatty acids and to bitter and sour solutions (Curtis et al., 2004; McDaid at al., 2007, Pittman et al., 2008; Wardwell et al., 2009). Taste sensitivity may change as well following adaptive physiological mechanisms to dietary requirements and nutrient imbalances (Brown and Toma, 1986; Leung et al., 1986). Animals reject diets that lead to amino acid depletion, deficiency or imbalance and select those that meet their requirements and have a positive postingestive effect (Edmonds et al., 1991; Koehnle et al., 2003; Kyriazakis et al., 1991; Owen et al., 1994; Gietzen et al., 2007).

In mammals, the impact of amino acids on gene expression has become an important area of research (Bruhat et al., 1999; Jefferson and Kimball, 2001; Averous et al., 2003). Mammals must adjust several physiological functions involved in the adaptation to amino acid availability by regulating the expression of numerous genes (Averous et al., 2003). Furthermore, in weaned piglets, gene expression is altered in small intestine at weaning and with dietary glutamine supplementation (Wang et al., 2008).

The aim of this study was to determine the pattern of expression of the porcine umami taste receptor genes, pT*as*1r3 and pT*as*1r1, in several tissues of the digestive system including tongue (circumvallate and fungiform papillae), stomach (fundus), pancreas, liver, duodenum, jejunum and ileum and how that pattern responds to three factors: (1) the age of the pig -from birth to 20 days after weaning-; (2) sex –male vs. female-; and (3) the dietary crude protein content and essential amino acid supplementation.

Materials and methods

All the animals used in this study were humanely managed according to the established guidelines of the European Union. The experimental protocol was approved by IRTA's Animal Care and Use Committee.

Housing

All the animals were housed in the experimental unit of the Department of Animal Nutrition IRTA-Centre Mas de Bover (Reus, Spain). Piglets were kept in the farrowing unit until day 26. At weaning, eighty pigs were housed in one weaning room (transition 3), equipped with automatic temperature control, forced ventilation and completely slatted floors. Feed and water were distributed ad libitum. Diets were mixed at IRTA's feed mill (IRTA Mas de Bover, Spain).

Animals and dietary treatments

Sixty-four piglets (Landrace x Pietrain) were used in this study, and were distributed into the following age groups: four 1 day-old piglets, four preweaned piglets (26 day old), eight 48hpostweaned piglets (28 day-old) divided into eater and non-eaters subgroups with four piglets each, and forty-eight 20-day postweaned piglets (46 day-old) that were randomly distributed into three dietary treatments (*Table 1*), with sixteen animals per diet to record performance data. These diets were defined by two levels of crude protein content of 26% (high crude protein –HCP- diet) or 17% (low crude protein diets). The low crude protein diets had either no addition of amino acids (LCP) or a supplementation of essential amino acids (SAA) to meet their amino acid requirements according to NRC (1998). All experimental feeds were medicated (Colistin sulphate, 80 mg*Kg^{-1}, Amoxicilin trihidrate, 300 mg*Kg^{-1}, and Copper sulphate, 140 mg*Kg^{-1}) and presented in mash form. Proximal analysis of crude protein (%), crude fiber (%), fat (%) and ash (%) contents were in accordance with the estimated nutrient composition (*Table 2*).

Animals were grouped based on a balanced distribution of weight, sex and littermates. The forty-eight 20-day postweaned pigs were divided into 4 initial body weight blocks and distributed in 12 pens at a rate of four replicates of four pigs per pen and dietary treatment. In the 48h postweaned group, half (eaters) were fed with the same feed offered to the HCP group although it was treated with chromium oxide (0.5%, green colour), as a faecal marker. The other half (non-eaters) did not have any access to feed during these first 48h after weaning. Each age group and treatment consisted of half males and half females.

Performance parameters

Body weight and feed intake were measured at weaning (BW0) and 20 days after weaning (BW20) and the average daily gain (ADG), average daily feed intake (ADFI) and feed to gain ration (F/G) calculated.

Table 1 > Distribution of dietary treatments at weaning depending on the dietary crude protein content.

TREATMENT[1]	CP LEVEL (%)	LYS LEVEL (g*Kg^{-1})	SYNTHETIC AMINO ACIDS
48h	26.00	15.15	D,L-Met
HCP	26.00	15.15	D,L-Met
LCP	17.00	8.97	No
SAA	17.00	14.03	L-Lys, L-Thr, D,L-Met, L-Trp, L-Ile, L-His,L-Val

1 - 48h: piglets from 48h after weaning with the HCP diet marked with 0.5% of chromium oxide. HCP: high crude protein diet. LCP: low crude protein diet. SAA: LCP diet supplemented with synthetic essential amino acids.

Tissue samples, RNA extraction and Real-Time RT-PCR for gene expression

Two male and two female piglets per age and dietary groups were anaesthetized using isofluorane (3-5%) and sacrificed by exsanguination. Two samples per tissue (one of 2g and one of 200mg) were excised, rapidly frozen in liquid nitrogen and maintained on a cryogenic freezer (-80ºC) for RNA extraction from each of the tissues studied: tongue (fungiform and circumvallate papillae), stomach (fundus), liver, pancreas, duodenum, jejunum and ileum. Total RNA from all pig tissues was extracted using Tripure® (Roche Applied Science, 11667165001) according to manufacturer's recommendations. Purity and quantity of RNA were analysed using the NanoDrop® N-1000 Spectrophotometer. The ratio of the readings at 260nm and 280nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. Selection criteria for all samples considered for analysis of RNA expression was set at A_{260}/A_{280} ratio of 1.80-2.00. The total RNA integrity and size distribution was also checked by denaturing agarose gel electrophoresis and ethidium bromide staining.

Table 2 > Composition of experimental diets (as fed basis).

INGREDIENTS (g*Kg^{-1}]	HCP	LCP	SAA
Corn	460.00	450.00	450.00
Corn starch	-	182.40	166.30
Soycomil	228.60	115.0	115.0
Whey sweet skimmed dehyd.	135.60	137.20	137.20
Soybean meal 48%	120.00	60.00	60.00
Lard	30.00	30.00	30.00
Dicalcium Phosphate	10.70	12.10	12.10
Calcium Carbonate	7.80	7.80	7.80
Mineral-vitamin premix [1]	4.00	4.00	4.00
Sodium chloride	1.40	1.50	1.50
D,L-Methionine	0.30	-	3.33
L-Threonine	-	-	13.10
L-Lysine HCl	-	-	31.7
L-Valine	-	-	8.10
L-Isoleucine	-	-	6.00
L-Tryptophan	-	-	4.10
L-Histidine	-	-	0.30
ESTIMATED NUTRIENT COMPOSITION[2]	HCP	LCP	SAA
Crude protein(g*Kg^{-1}]	260.00	166.90	170.00
Crude Fiber(g*Kg^{-1}]	22.80	16.60	16.60
Fat(g*Kg^{-1}]	50.20	49.00	49.00
Lactose(g*Kg^{-1}]	100.00	100.00	100.00
Ash(g*Kg^{-1}]	59.40	50.00	50.00
Lysine(g*Kg^{-1}]	15.53	9.70	14.03
Methionine(g*Kg^{-1}]	4.21	2.98	5.72
Threonine(g*Kg^{-1}]	10.80	6.79	9.16
Tryptophan(g*Kg^{-1}]	2.97	1.92	2.52
Energy (Kcal ME*Kg^{-1}]	3572	3522	3538

1 - contains vitamins and minerals, 140 mg*Kg^{-1} Copper Sulphate, 300 mg*Kg^{-1} Amoxicilin Trihidrate and 80 mg*Kg^{-1} Colistin Sulphate. Vitamins and minerals per Kg of diet: Vitamin A, 10000 IU,; Vitamin D3, 2000 IU; Vitamin E 15mg; Vitamin B1, 2 mg; Vitamin B2, 7 mg; Vitamin B12, 0.030 mg; Vitamin K3, 2 mg; D-calcium Pantothenate, 30 mg; Nicotinic acid, 50 mg; Biotin, 0.1 mg; Folic acid, 1.2 mg; Fe, 70 mg as iron carbonate; Cu, 165 mg as copper sulphate pentahydrate; Co, 0.14 mg as cobalt carbonate; Zn, 125 mg as zinc oxide; Mn, 43 mg as a manganese sulphate; I, 0.74 mg as potassium iodate; Se, 0.30 mg as sodium selenite; Etoxiquin, 100 mg. 2 - Proximal analysis of Crude Protein (%), Crude Fiber (%), Fat (%) and Ash (%) contents were corresponded to the estimated nutrient composition.

Table 3 shows the primers for pT*as*1r1 and pT*as*1r3 primers designed using Primer Express software version 2.0 (Applied Biosystems) and primers from the selected pig housekeeping gene (Tata Box binding Protein, TBP) were those referred by Nygard et al. (2007). These primers were used at optimized concentrations of 900nM for pT*as*1r3 and TBP forward primers and 300nM for pT*as*1r1 forward and pT*as*1r1, pT*as*1r3 and TBP reverse primers. First strand cDNAs were synthesised from 1µg of total RNA using the Quantitect reverse transcription kit® (Qiagen, 205311). Real-Time PCR analysis was performed in triplicates using the Fast SYBR Green Master Mix® (Applied Biosystems, AB0155) and the ABI 7500 Fast Real Time PCR system (Applied Biosystems). Thermal cycling parameters for the real-time PCR were as follows: 95ºC for 20s, followed by 40 cycles of 95ºC for 3s and 60ºC for 30s. The dissociation curve was analysed in every plate, and its thermal cycling parameters were as follows: 95ºC for 15s, 60ºC for 1 min., and 95ºC for 15s. Values for cycle thresholds (Ct) were calculated by the Applied Biosystems software. The Ct value indicates the number of cycles to the exponential growth of the PCR product during the log-linear phase. Calculation of the starting mRNA's concentrations and individual PCR efficiencies for each sample were calculated by LingReg PCR software version 7.0 (Ramakers et al., 2003).

Standard PCR amplicons from tongue samples were developed using Platinum Taq DNA polymerase high fidelity kit® (Invitrogen, 11304-011). DNA was extracted using QIAquick gel extraction kit® (Qiagen, 28704) using a microcentrifuge. Amplicons were sequenced to confirm that the bands corresponded to pT*as*1r1 and pT*as*1r3 genes (Davis Sequencing, 1490 Drew Avenue, Suite 170, Davis, CA 95618, USA).The thermal cycling for the standard PCR used to produce the sequencing amplicons were as follows: 94ºC for 2 min., followed by 41 cycles of 94ºC for 30s, 57ºC for 30s and 60ºC for 10s, and 68ºC for 7min.

Table 3 > Selected primers for pT*as*1r1, pT*as*1r3 (target genes) and pig TBP (housekeeping gene) genes.

GENE	FORWARD (5'->3')	REVERSE (5'->3')	AMPLICON LENGTH (bp)
TBP	AACAGTTCAGTAGTTATGAGCCAGA	AGATGTTCTCAAACGCTTCG	153
pT*as*1r1	ATCTGTTCTCGAGGCCAAGTCT	GCGAGTCCCCACTGTCACTAA	159
pT*as*1r3	CGCAGCATTGCCACCTACTG	TAGCTGACCTGCGGCATGAG	134

Calculations of gene expression

A relative quantification of fold changes in gene expression was performed to study changes in the porcine umami taste receptor genes. The relative quantification relies on the comparison between expression of a target gene versus a reference gene (housekeeping) and the expression of the same gene in a target (experimental) sample versus a control sample (Pfaffl, 2001). The relative expression of target genes were determined on the basis of the Cycle threshold (Ct) values from target genes that were adjusted by Ct readings of a housekeeping gene to prevent any potential unaccounted variation or bias (Fu et al., 2006). The Ct values permit to calculate the delta-delta Ct ($\Delta\Delta$Ct), and the term $\Delta\Delta$Ct measures the relative change of expression of the target gene from experimental to control samples compared to the reference gene, where the first delta comes from Ct_{target}- $Ct_{reference}$ of the control sample minus $Ct_{ttarget}$-- $Ct_{reference}$ of the experimental sample (Fu et al., 2006). Finally, the fold change was derived from $2^{-\Delta\Delta Ct}$ (Livak et al., 2001) and fold change means the amount of signal in the experimental sample compared to the control sample for the target gene after housekeeping gene normalization.

The relative expression of our target genes (pT*as*1r1 and pT*as*1r3) were determined on the basis of the Ct values that were adjusted by Ct readings of a housekeeping gene (TBP). Liver pT*as*1r1 and pT*asr*3 gene expression at preweaning was selected as the reference value to study age and dietary treatment effects in each gene. The liver was used as reference value relative to other tissues. The pT*as*1r3 gene expression was used as reference value relative to pT*as*1r1 gene expression and male was the reference value relative to female gene expressions.

Statistical analysis

The Ct values were analyzed using the generalized estimating equations (GEE) model including an exchangeable correlation structure using the GENMOD procedure in SAS (Fu et al., 2006), with the number of replicates as repeated measurements according to the following models:

$$Y_{il} = \mu + \alpha_i + \omega_1 + \varepsilon_{il} \ (\text{age effect})$$
$$Y_{jl} = \mu + \beta_j + \omega_1 + \varepsilon_{jl} \ (\text{tissue effect})$$
$$Y_{kl} = \mu + \delta_k + \omega_1 + \varepsilon_{kl} \ (\text{diet effect})$$

where Y_{il}, Y_{jl} and Y_{kl} are the dependent variable for the observation or delta-delta Ct value ($\Delta\Delta$Ct) at every age (α_i=birth, 26d-old, 48h postweaning and 20 days

postweaning under HCP diet); for every tissue (β_j=Tongue fungiform and circumvallate papillae, stomach, liver, duodenum, jejunum and ileum); in every dietary treatment (δ_k= 20 days postweaning HCP, LCP and SAA); and in each sex (ω_l=males, females); μ is the general mean for all observations; and ε_{jl} and ε_{jl}, and ε_{kl} is the residual error. Least square means were separated into significant main effects by the DIFF option in SAS. P-values <0.05 were considered significant.

The ΔΔCt values (Mean ± SEM) and their 95% confidence intervals for every target gene (pT*as*1r1 and pT*as*1r3) were obtained for each gene, age, tissue, dietary treatment and by sex. Finally, the fold change was derived as $2^{-\Delta\Delta Ct}$ (Livak et al., 2001). Fold change results were described in a logarithmic transformation and expressed as Mean±SEM.

Performance data (BW0, BW20, ADG, ADFI and FG) were analyzed using the GLM procedure in SAS, with dietary treatment and live weight as main effects. For statistical analysis, a randomized block design was used. Mean values for the effect of treatment on performance parameters were compared using the GLM procedure and the Tukey adjustment in SAS. P-values <0.05 were considered significant.

All the statistical analyses were performed using the Statistical Analysis System (version 9.1., Software SAS Institute Inc., Cary NC, 1991-2001).

Results

Performance parameters during the prestarter stage

Overall performance results from weaning to 20 days post-weaning are shown in *Table 4*. The overall ADG, ADFI and F/G of the control group (HCP) were 226 and 282g/d and 1.24, respectively. Significant diet effects were found in BW20 and ADG (P<0.05). Pigs fed the SAA and LCP diet grew less (179.58 and 155.28g/d, respectively) than the control group (HCP). On the other hand, no differences were showed in ADFI and FG, although conversion rate was numerically worse in LCP than HCP and SAA groups.

Table 4 > Performance parameters of piglets during the prestarter stage (from weaning to 20 days after weaning) fed with different diets (High crude protein- HCP-, Low crude protein –LCP- and Low crude protein supplemented with essential amino acids –SAA-).

DIETS[1]	BW0 (g)[2]	BW20 (g)[2]	ADG (g*day^{-1})[2]	ADFI(g*day^{-1})[2]	FG[2]
HCP	8448	12985[a]	226.82[a]	281.70	1.24
LCP	8433	11539[b]	155.28[b]	263.93	1.70
SAA	8337	11929[ab]	179.58[b]	246.40	1.37
Root MSE	474.08	1180.23	50.06	92.47	1.16
Diet effect	0.7872	0.0055	0.0014	0.8673	0.3204
Block effect	<0.0001	<0.0001	<0.0001	0.1508	0.4234

1 - The average measurements of each pen (4 piglets/pen) were used to calculate the mean values of ADFI and FG for each treatment (data from 16 animals/treatment) / **2** - Different letters mean significant differences (P<0.05). Pig umami TasR expressions in taste and non-taste tissues

Pigs from the 48h postweaned group were not separated into eaters and non-eaters. None of them ate during these first 48h. No marked faeces in their pens were recorded and no gastric content was found in euthanized animals.

The samples of pancreas from all animals and jejunum from LCP group were excluded as they had an A_{260}/A_{280} ratio lower than 1.70 (presence of impurities) and the respective ribosomal bands (28S and 18S) did not appear as sharp bands on the stained gel that is indicative of RNA degradation during sample preparation. Selected samples had the A_{260}/A_{280} ratio between 1.80 and 2 and an intact rRNA subunit of 28S and 18S on the gel indicating minimal degradation of the RNA. Sequence analysis confirmed that PCR products of the predicted size were 100% homologous to the pig sequences cloned from the circumvallate papilla on the tongue showing that the pT*as*1r1/pT*as*1r3 are expressed in both taste and non-taste tissue samples.

The Tata Box binding Protein (TBP) gene was used as housekeeping gene. The TBP Ct was within 24.39 and 29.69. The pT*as*1r1and pT*as*1r3 Ct's were 31.83±2.14 and 31.73±1.91, respectively.

PTas1r1 gene expression relative to pTas1r3 gene expression

No differences (P>0.05) where observed in the pTas1r1 gene expression relative to pTas1r3 with age and with dietary treatment. Therefore, the mean of the data from all ages and dietary treatments was used to analyse differences in pTas1r1 gene expression relative to pTas1r3 in every tissue. The pTas1r1 showed significantly (P<0.05) lower levels of expression than pTas1r3 in stomach and jejunum (*Figure 1*). The high variability within the same tissues may be partially due to the effect of age, sex and diet.

Figure 1 > The pTas1r1 fold change vs. pTas1r3 gene expression in different tissues expressed in a log scale (Mean±SEM). Each column represents the mean of the four ages (birth, 26, 28 and 46 days old) and dietary treatments (HCP, LCP and SAA).
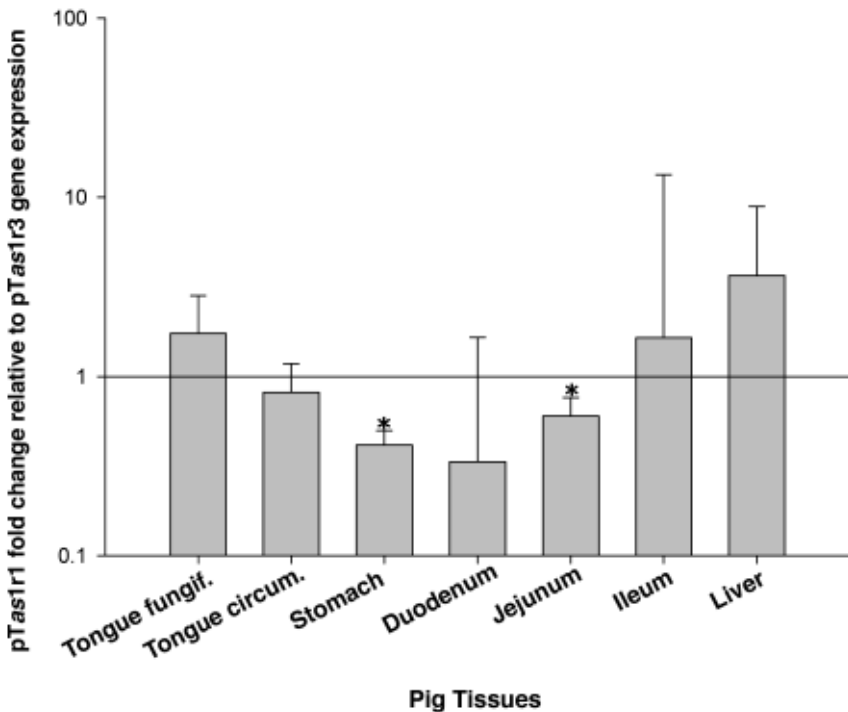
***** Columns with an asterisk show significant differences (P<0.05).

PT*as*1r1 and pT*as*1r3 gene expressions in different tissues with age relative to their expression in liver before weaning

Both genes were expressed at all ages (Birth, 26d-old, 48h and 20d fed with high dietary protein –HCP- after weaning) and in all tested tissues (*Figures 2 and 3*). Age dependent differences were observed in both gene expressions pT*as*1r1 (P<0.05) and pT*as*1r3 (P=0.08). The expression of the pT*as*1r1 (*Figure 2*) was age dependent in duodenum (P<0.05) and with trends (P=0.07) in jejunum and ileum but without any apparent common pattern. Age dependency for the pT*as*1r3 (*Figure 3*) was found in the jejunum (P<0.05) with trends (P=0.09) in the tongue fungiform papillae, duodenum and ileum. Both genes were upregulated in jejunum just 48h after weaning (*Figure 2 and 3*).

Figure 2 >The pT*as*1r1 fold change in different tissues with age relative to pT*as*1r1 expression in the liver before weaning expressed in a log scale [Mean±SEM][1,2].
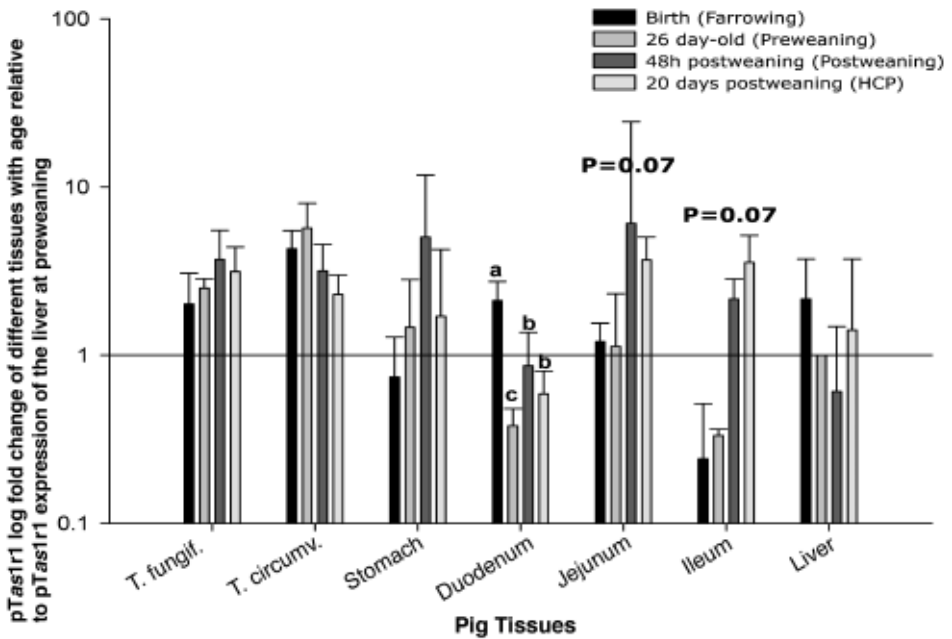
1 - Different letters mean significant differences (P<0.05) within the same tissue. / 2 - Statistical trends are specified by their P value only when lower than 0.1.

Figure 3 > The pT*as*1r3 fold change in different tissues with age relative to pT*as*1r3 expression in the liver before weaning expressed in a log scale (Mean±SEM) [1,2].
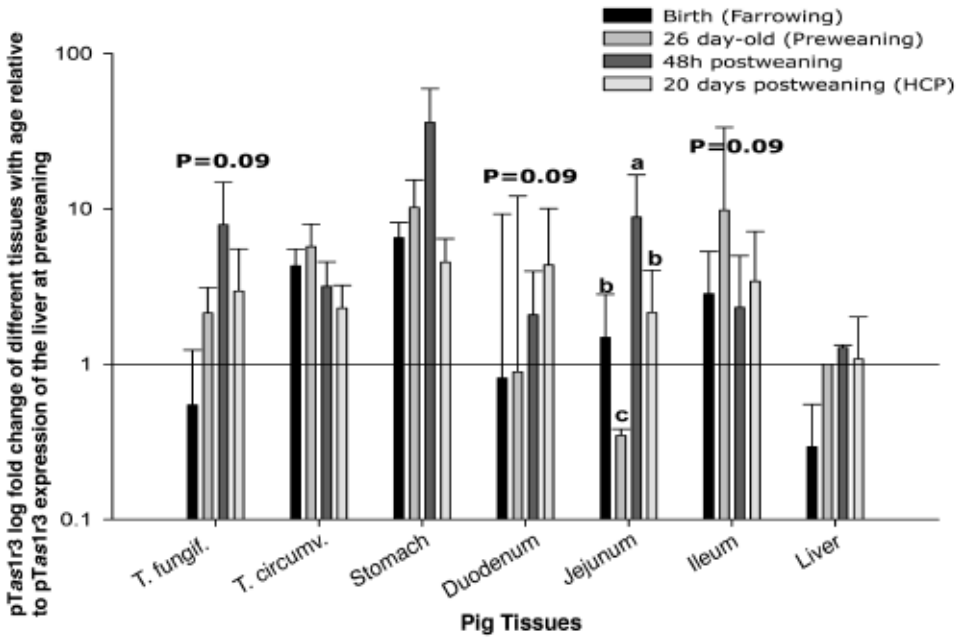
[1 - Different letters mean significant differences (P<0.05) within the same tissue. / 2 - Statistical trends are specified by their Pvalue only when lower than 0.1.]

The pT*as*1r3 was also upregulated in the tongue fungiform papillae (*Figure 3*). In taste tissues and stomach both genes had similar patterns of expression by age (*Figures 2 and 3*).

Differences of expression between sexes (data not shown) were also age-dependent. The pT*as*1r1 had a higher expression in males than in females at birth and 20d after weaning (P<0.05) and at 48h after weaning (P=0.06) while the pT*as*1r3 was apparently more expressed in males than in females at 48h after weaning (P=0.06) and 20d after weaning (P=0.08).

PT*as*1r1 and pT*as*1r3 gene expressions in different tissues relative to their expression in the liver

Figure 4 shows the expression of both genes in every tissue as a mean of their log fold change of expression relative to the liver across experimental periods.

Figure 4 > The pT*as*1r1 and pT*as*1r3 log fold changes in different tissues relative to their expression in the liver, expressed in a log scale (Mean±SEM). Each column represents the mean of the four ages (birth, 26, 28 and 46 days old) in every tissue.
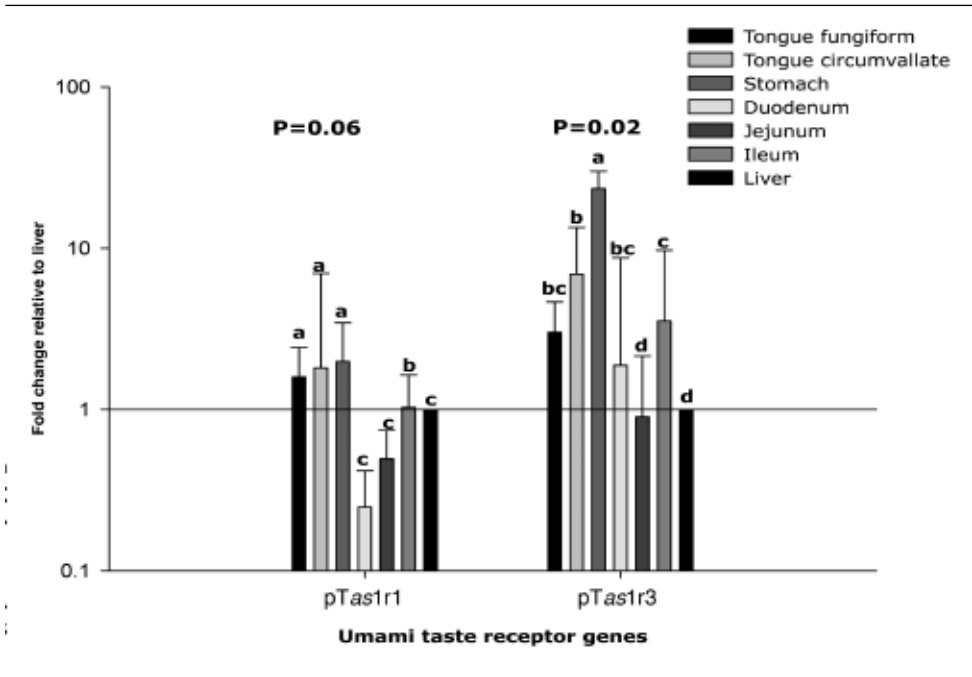
1- Different letters mean significant differences (P<0.05) within the same tissue. / 2 - Statistical trends are specified by their P value only when lower than 0.1.

The pT*as*1r1 gene had a higher expression in taste tissues and stomach than small intestine and liver (P=0.06) whereas the pT*as*1r3 gene had a higher expression in stomach (P<0.05), followed by taste tissues, small intestine and liver.

Both genes showed a significant (P<0.05) sex effect in different tissues (Data not shown) with males having a higher level of expression than females. Tissues with sex dependent expression were tongue circumvallate and fungiform papillae, duodenum and jejunum for the pT*as*1r3 and only tongue fungiform papillae for the pT*as*1r1.

Effect of dietary protein level and amino acid supplementation on umami T*as*R expression

The pT*as*1r1 had a higher (P<0.05) expression in the duodenum of the LCP group than duodenum samples of the SAA and HCP groups (*Figure 5*).

Figure 5 > The pT*as*1r1 fold change in different tissues with dietary treatment relative to its expression in the liver before weaning expressed in a log scale (Mean±SEM).
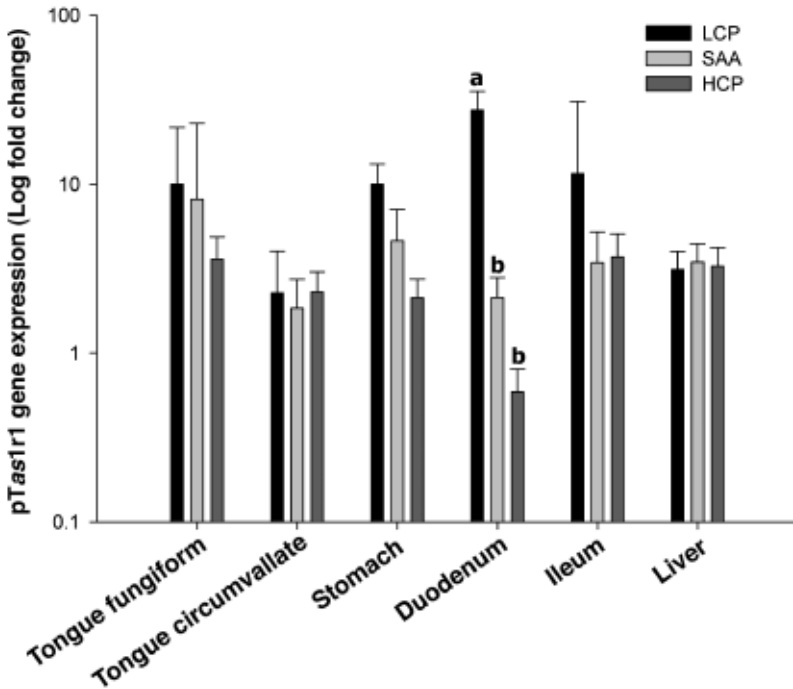
Different letters mean significant differences (P<0.05) within the same tissue.

The pT*as*1r3 did not show any significant difference. Both genes showed numerical increase in their expression in tongue fungiform, stomach and ileum with the LCP diet compared to the SAA and HCP diets. The LCP group jejunum samples were lost and data from the other treatments on jejunum were discarded for analysis.

Finally, duodenum of LCP and SAA groups had significant differences in expression of both genes by sex (P<0.05). Females showed higher levels of expression than males (data not shown).

Discussion

In intensive pig production, weaning is the most critical period with important consequences on its performance (King and Pluske, 2003). In piglets, weaning is associated with reduced food consumption, intestinal dysfunction and atrophy (Cranwell, 1995; Miller and Slade, 2003) but also with the alteration of gene expression in the small intestine (Wang et al., 2008). Dietary amino acids have an important role in the regulation of gene expression. Depletion or a dietary imbalance of some amino acids or an insufficient intake of protein can induce gene expression changes (Bruhat et al., 1999; Jefferson and Kimball, 2001). The radical dietary change at weaning contributes to cause a lack of appetite resulting in an insufficient supply of energy and protein for maintenance requirements (Le Dividich and Sève, 2001; Bruininx et al., 2002; Brooks and Tsourgiannis, 2003). Thus, an insufficient protein intake usually occurs in piglets just after weaning and can promote gene expression changes.

On the other hand, newly weaned piglets depend mostly on their sensorial capacity and previous experience (social learning) to assess the nutritional or toxicant content of novel feeds (Forbes, 1998; Bolhuis et al., 2009). Pigs perceive some L-amino acids through the porcine umami taste receptor, pT1r1/pT1r3 (Chapter 2), indicating the presence of protein (Ninomiya, 2002). Therefore, it may be expected that pTas1r1 and pTas1r3 gene expressions are altered by dietary crude protein content or insufficient protein supply such as at weaning.

We designed three experimental diets (from weaning to 20 days after) that differed in dietary protein content to study possible changes in the expression of the porcine umami taste receptor genes. The experimental crude protein level (%) for the control diet was set at 26 (HCP diet) to meet or exceed the amino acid requirements without the addition of purified amino acids, with the exception of D,L-Methionine that was added at 0.3g*Kg^{-1} (NRC, 1998). In contrast, a diet was set at 17% crude protein level (LCP diet) with a low level of protein that theoretically does not meet the essential amino acid requirements of growing pigs at this stage without the addition of purified amino acids (NRC, 1998) and finally, the LCP diet supplemented with essential amino acids (SAA diet) to reach similar levels of essential amino acids than in the HCP diet.

Pigs are able to detect added purified amino acids through organoleptic properties (Ettle and Roth, 2004; Ettle et al., 2005; Roth et al., 2006) and their postingestive

consequences may determine consumption or avoidance of such diets (Gietzen, 1993; Koehnle et al., 2003; Ettle and Roth, 2004). The addition of purified amino acids would likely have an impact on feed palatability, although our porcine umami cell reporter system (Chapter 2) did not show activation of the pT1r1/pT1r3 taste receptor by high levels of essential amino acids (EAA). Therefore, we may expect similar responses in their expression with SAA and LCP diets.

Performance results showed significant differences in growth and final body weight among diets, without a significant difference in feed intake (*Table 4*). All diets were isoenergetic and differed in their dietary protein content or amino acid profile (*Table 1*). The SAA and HCP diets had similar EAA profile compared to the LCP diet and the SAA and LCP diets differed in their non-essential amino acid (NEEA) profile and in their dietary crude protein content compared to the HCP diet. The low values of ADG and BW at 20 days post-weaning in all treatments may result from a lack of adaptation of the animals to the experimental feeds, particularly during the first week after weaning. It was observed that animals in all the dietary groups did not consume feed until day 3 after weaning. Such a long anorexia period across treatments would have minimized most of the expected differences in productive parameters such as in FG among the HCP, LCP and SAA groups.

The L-Glutamic acid (L-Glu) and L-Glutamine (L-Gln) are pig umami tastants present in high amounts in pig diets with several physiological and metabolic roles in the body (Bellisle, 1999; Reeds et al., 2000; Duggan et al., 2002; Nakamura et al., 2008). Both are NEAA although L-Gln is considered a conditionally essential amino acid in stressful conditions, particularly as it relates to supporting the metabolic requirements of the intestinal mucosa when glucose depletion is severe and/or when the intestinal mucosa is damaged by starvation (Kim et al., 2007). The L-Glu and L-Gln are extensively oxidized in the small intestine epithelial cells and this oxidative capacity coincides with a high energy demand of the epithelium, which is in rapid renewal and responsible for the nutrient absorption process (Blachier et al., 2009). As mentioned before, the weaning process causes a reduction of feed intake in pigs that produces damage in the intestinal mucosa. These diets were offered from weaning to 20 days after weaning and in this stage we should consider a high requirement of nutrients (such as L-Glu and L-Gln) that functions as fuel for enterocytes. The high amounts of these amino acids in the HCP diet may explain part of the improvement of piglet's growth in this group compared to SAA and LCP diets. Wang et al. (2008) suggested that dietary supplementation of glutamine prevents intestinal dysfunction and atrophy, promotes small intestine growth and improves body weight gain in weanling piglets. On the other hand, the fact that there were no significant differences

in ADFI may suggest that pigs have their energy and protein maintenance requirements covered. Therefore, we may consider that there is not an excess or deficiency of amino acids that decreases their feed consumption (Kyriazakis et al., 1991; Gietzen, 1993; Gietzen et al., 2007). Feed intake reduction and growth depression were evident in pigs fed diets deficient in an EAA or with an excess of them (Edmonds et al., 1987; Ettle and Roth, 2004; Gietzen et al., 2007).

Pigs perceive amino acids by the umami G-protein coupled-receptor heterodimer T1R1/T1R3 located in taste bud cells (Chapter 2) and these umami tastants are highly preferred by pigs (Chapter 1). The pT*as*1r1 and pT*as*1r3 gene expressions in the tongue showed that the sensory mechanism to identify nutrients starts in taste bud cells, allowing pigs to detect pleasant compounds on diets. Yasuo et al. (2008) proposed that the signal mediated by the pathway involving T1R1/T1R3 taste receptor plays an important role in preference behaviour in the anterior tongue.

Apart from the oral cavity, T1R1/T1R3 umami taste receptor and even their transduction molecules were found in non-taste tissues like stomach and gut mucosa of rodents and humans (Dyer et al., 2005; Bezençon et al., 2007; Mace et al., 2007). Dyer et al. (2005) also determined the pattern of expression of mT*as*1r3 and mT*as*1r1 genes in the mouse small intestine. The mT*as*1r3 was slightly higher in jejunum than in duodenum and ileum, and mT*as*1r1 was higher in ileum than in duodenum and jejunum. They suggested that both receptors may be expressed in subpopulations of cells along the small intestine, with different pattern of expression between T1R1 (higher in the crypth fractions) and T1R3 (higher in the villous cell fractions). Our results showed that both porcine umami taste receptor genes were expressed along the GI tract (tongue fungiform and circumvallate papillae, stomach, duodenum, jejunum, ileum and liver). Another study (Kiuchi et al., 2006) in three and a half month-old male pigs also found the pT*as*1r3 expressed in different tissues of the gastrointestinal (GI) tract. These results suggest that pigs have chemoreceptor cells along the GI tract that are sensing protein-born compounds of the luminal content.

We selected the TBP as a housekeeping gene to normalise our data. The TBP is one of the most stable genes across tissues and it is an especially interesting as a good reference gene for low abundant transcripts (Nygard et al., 2007). In our results, TBP in all tissues ranged between 24.39 and 29.69 Ct values. For all tissues the pT*as*1r1 and pT*as*1r3 Ct were high (31.83±2.14 and 31.73±1.91, respectively) showing that these genes had low expression levels in our pig tissues. The expression of T1R's in mice also showed low levels of their expression in the tongue and similarly in other tissues from the GI tract (Dyer et al., 2005).

The quantitative results were expressed as the relative comparison with the liver pT*as*1r1 and pT*as*1r3 expression in preweaned pigs mainly. The liver showed the lowest levels of expression in both genes, as it was previously observed by Kiuchi et al. (2006) for pT*as*1r3. Preweaning was the selected age as it was considered to be the least stressful of the periods under study. At birth, for example, the newborn piglet experiences dramatic changes in its thermal environment and its source of energy and nutrients (Herpin and Le Dividich, 1995), whereas at weaning (48h after weaning) and at the prestarter stage (20-d postweaning) there are several social, nutritional and environmental sources of intense stress (Pluske et al., 1995). The expression values referred to a single value (preweaning liver levels) allowed us to compare the variation of the level of the porcine umami taste receptor among different ages and dietary treatments. In order to compare differences in gene expressions between pT*as*1r1 and pT*as*1r3 genes, differences between males and females and among tissues, the pT*as*1r3 gene expression, male and liver were considered as control samples, respectively.

Our results showed that both genes were expressed similarly in tongue fungiform and circumvallate papillae at all ages and regardless dietary treatments (*Figure 1*). Since the umami taste receptor heterodimer (T1R1/T1R3) shares the T1R3 subunit with the sweet taste receptor heterodimer (T1R2/T1R3), we hypothesized that pT*as*1r3 would be more expressed than pT*as*1r1 (Li et al., 2002; Nelson et al., 2002). Our results showed that both genes had similar levels of expression except in the stomach and in the jejunum, where pT*as*1r1 was less expressed than pT*as*1r3 (*Figure 1*). Therefore, higher levels of expression of pT*as*1r3 compared to pT*as*1r1 would have suggested the presence of the sweet taste receptor dimer (T1R2/T1R3) or tentatively the function of the T1R3 as a monomeric or homodimeric taste receptor (Damak et al., 2003; Zhao et al., 2003).

The pT*as*1r1 (P=0.06) and pT*as*1r3 (P=0.02) showed differences in their expression by tissues (*Figure 4*). In our pigs, similarly to Kiuchi et al. (2006) with their pT*as*1r3 results, both genes were more expressed in tongue and stomach than small intestine and liver (*Figure 4*). The fundic region of the stomach has the oxyntic cells that secrete hydrochloric acid, and the mucous cells that secrete proteases. The detection of umami tastants in the tongue and stomach initiates digestive responses such as cephalic responses of pancreatic secretions (Ohara et al., 1988; Niijima at al., 1990; Naim et al., 1991) and gastric secretion and motility (Uneyama et al., 2006; San Gabriel et al., 2007; Zai et al., 2009). Therefore, the presence of both genes in these tissues may be more related to identification and digestion of sources of protein than absorption and metabolism of amino acids.

On the other hand, the pT*as*1r3 gene expression was higher than pT*as*1r1 gene expression (P<0.05) only in the stomach and jejunum (*Figure 1*), probably due to the presence of the sweet taste receptor heterodimer or as taste receptor by itself in these non-taste tissues (Li et al., 2002; Nelson et al., 2002; Damak et al., 2003; Zhao et al., 2003).

There are changes in taste perception with age in humans and rodents (Stevens and Cain, 1993; Drewnowski, 1997; Tordoff, 2007). We found that the pT*as*1r1 and pT*as*1r3 gene expressions in the pig tongue, stomach and liver did not vary with age (*Figure 2 and 3*). Nevertheless, both genes seemed upregulated in the tongue fungiform papillae and stomach just after weaning. The upregulation of both genes just after weaning in these tissues may suggest that the pig umami taste receptor is indicative of their low protein status due to weaning anorexia.

In some parts of the small intestine both genes also increased their expression, but after 20 days after weaning. These findings may involve these genes in nutrient absorption processes in this stage. The expression of pT*as*1r1 was higher in the jejunum and ileum (P=0.07) than in the other tissues whereas the expression of pT*as*1r3 seemed higher in the duodenum (P=0.09) and jejunum (P<0.05) (*Figure 2 and 3*). There are several physiological responses of the GI tract at weaning that influence nutrient absorption, such as changes in intestinal morphology due to villous atrophy and crypt hyperplasia that may cause a transitory reduction in amino acid absorption capacity (Miller and Slade, 2003). Postweaning repair and development of intestinal structure and function involve high demands by enterocytes of amino acids such as glutamine and glutamate, (Miller and Slade, 2003) that have been described as pig umami tastants (Chapter 2). Glutamate and glutamine are the most important source of metabolic fuel for the mucosa and the preferred fuel for enterocytes, respectively (Stoll et al., 1999; Burrin and Stoll, 2003). Wang et al. (2008) showed that gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. These authors concluded that there were coordinate alterations of gene expression in response to weaning and suggested molecular mechanisms for the beneficial effect of dietary glutamine supplementation on nutrition status in weaned piglets. Therefore, the porcine umami taste receptor in the small intestine may play a role in nutrient absorption processes and its upregulation may identify low amino acid levels in the lumen after weaning.

The expression of the porcine umami taste receptor seemed also to be influenced by the dietary protein content. The pT*as*1r1 gene expression was clearly upregulated in the duodenum of the LCP group (*Figure 5*). No significant differences were showed

in other tissues among dietary treatments in both genes, although those seemed upregulated in tongue fungiform papillae and stomach of the LCP and SAA groups compared to the HCP group (*Figure 5*). Again, the upregulation of these genes may explain a low protein status in these piglets due to low crude protein content in the experimental diets of these groups. We may suggest that the LCP and SAA diets were insufficient in protein to provide the amino acid requirements to rapidly repair and develop the intestinal villous after weaning. Consequently, nutrient absorption may be reduced, and in these circumstances the expression of the porcine umami taste receptor may increase for improving the luminal sensing capacity of pigs towards specific amino acids used as fuel for enterocytes.

The regulation of the expression of taste molecules depending on nutrient status has been previously described in rats, showing a link between the sweet (T1R2/T1R3) and the amino acid (T1R1/T1R3) taste receptors in nutrient absorption processes (Mace et al., 2009). The L-Glu causes in the jejunum the rapid internalization of T1R1, T1R3 and transducin through cytosolic vesiculation, whereas it causes apical membrane insertion of T1R2, α-Gustducin, PLC β2 and PKC βII (Tan et al., 2004; Scherrer et al., 2006; Mace et al., 2009). The physiological significance of this coordinated regulation seems to be the control of energy supply for enterocytes, mainly that of glucose and NEAA (L-Glu and L-Asp). Young et al. (2009) observed a negative correlation between the expression of taste molecules (T1R2, T1R3, TRPM5 and Gustducin) and the glucose plasma level in the GI tract of humans, and between the level of expression of T1R2 and the luminal content of glucose in the jejunum in mice. Similarly, our results showed that both genes seemed to be upregulated due to an insufficient feed protein intake (48h after weaning) or to low dietary crude protein content (20d after weaning with the LCP and SAA diets). These situations may reduce the luminal content of umami tastants, mainly NEAA that are nutrients for enterocytes, the requirements of which may increase with villous atrophy. Therefore, upregulation of pT*as*1r1 and pT*as*1r3 genes may reveal a low protein status and a high demand of nutrients by the intestinal epithelium in weaned pigs and their expression may also evaluate the effect of dietary umami tastants in pig nutrition status.

Sex differences in taste responses were described in several studies (Curtis et al., 2004; McDaid at al., 2007, Pittman et al., 2008; Wardwell et al., 2009). For example, in rats, females were less sensitive to NaCl and sucrose solutions than males (Curtis et al., 2004) and in pigs, gilts had a higher preference for high-lysine diet than males (Owen et al., 1994). Our results showed that males normally had higher levels of expression than females after weaning, although females showed higher expression in

the duodenum of the LCP and SAA groups. We hypothesize that males respond more dramatically to weaning (increasing the pT*as*1r1 and pT*as*1r3 gene expressions) than females to improve their amino acid-sensing capacity in taste tissues, whereas females would respond more than males to a depletion of dietary protein during the prestarter stage at the intestinal level. The sex effect explains part of the high variability observed in the umami taste receptor expression and this trend merits further investigation.

Finally, we conclude from our data that the detection of umami tastants in pigs starts in the tongue and continues along the GI tract through the activation of the pT*as*1r1/pT*as*1r3 taste receptor. Therefore, the stimulation of the porcine umami taste receptor play a significant role in the intake, digestion and absorption of nutrients, and its expression in the GI tract may change with age (weaning), sex and dietary protein content.

Conclusions

The porcine umami taste receptor T1R1/T1R3 is expressed in taste tissues (tongue circumvallate and fungiform papillae). Apart from the tongue, this receptor is present in other tissues of the GI tract in pigs, mainly in stomach and small intestine. These organs may be using this chemosensory mechanism to sense umami tastants in the GI lumen. This receptor is fully developed at birth and its expression changes with age (weaning), sex and dietary protein content depending on the tissue. The pT*as*1r1 and pT*as*1r3 genes may respond increasing their expression at weaning, with a low feed consumption, high requirements of umami tastants by enterocytes and with low dietary protein feeds that do not provide enough nutrients for villous construction and development. Umami tastants are firstly detected by the porcine umami taste receptor in the tongue and their identification may continue along the GI tract, and its activation may play a role in nutrient digestion and absorption processes. A proper knowledge of the changes in the expression of the umami taste receptor would help to develop new strategies for improving the adaptation of piglets to the postweaning period by using potential umami tastants that stimulate voluntary feed intake.

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General Discussion

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Introduction

One of the most important priorities in piglet nutrition is to prevent weaning anorexia or low levels of feed and water intake just after weaning. The radical dietary change at weaning contributes to cause a lack of appetite, sub-optimal growth rate and diarrhea (Vente-Spreeuwenberg and Beynen, 2003). In addition, weaning is associated with changes in small intestinal integrity such as villous atrophy and the decline of digestive enzymes reducing the capacity of nutrient digestion and absorption as a consequence of the decreased feed intake and low supply of intestinal nutrients in the first post-weaning days (McCracken et al., 1999; King and Pluske, 2003). As digestion and absorption are impaired, the presence of unabsorbed nutrients at ileal and colonic levels increases and finally results in secretion of anorexic signals like Glucagon-like Peptide-1 (Cranwell, 1995; Pluske et al., 1997; Miller and Slade, 2003). The maintenance of enteric health and intestinal function is a key for supporting growth of the weaned pig (Burrin and Stoll, 2003). Therefore, dietary strategies to prevent the negative effects of weaning should focus on the use of compounds that stimulate food preference and consumption in weanling piglets combined with functional feed ingredients to improve gut morphological and functional adaptation in the post weaning phase.

Newly weaned piglets depend mostly on their sensorial capacity and previous experience (social learning) to assess the nutritional or toxicant content of novel feeds (Forbes, 1998; Bolhuis et al., 2009). Sensory perception of taste, odor, and texture influences food preference and consumption (Amarantos et al., 2001; Drewnoski et al., 2001; Wardwell et al., 2009). Animals recognize the nutritional quality of feed and feedstuffs partially through the basic tastes (sweet, bitter, salty, acid and umami). A positive taste (mainly sweet, salty and umami) will stimulate appetite for ingredients while a negative taste (bitter and possibly sour) will lead to rejection of harmful substances.

Protein is the most satiating macronutrient and is associated with peripheral and central signaling processes involved in food intake control (Potier et al., 2009). Preferences for different sources of protein and levels of a particular amino acid have been studied by choice-feeding trials in pigs, showing that piglets are able to choose a balanced combination of protein sources or amino acids that maximizes their performance (Kyriazakis et al., 1990; Morgan et al., 2003; Ettle and Roth, 2004; Ettle and Roth, 2005; Roth et al., 2006; Solà-Oriol, 2008). The umami taste is a pleasant taste (Bellisle, 1999) that indicates the presence of protein (Ninomiya, 1998). There are specific taste receptors that identify umami ligands, such as monosodium glutamate (Li et al., 2002; Nelson et al., 2002). The heterodimer receptor T1R1/T1R3 is considered to be a promiscuous amino acid-sensing receptor present in the tongue and in non-taste tissues that responds broadly to Gly and L-amino acids (L-AA). Its response is enhanced by purine ribonucleotides like 5'-inosine monophosphate (IMP) (Wellendorph et al., 2009). Its promiscuity in relevant tissues allows this receptor to play a role in nutrient-sensing and other physiological processes (Sbarbati et al., 2009). We hypothesized that potential umami response in pigs, such as in other mammals, may help to recognize sources of protein and may stimulate feed intake at weaning preventing some of the problems previously described.

The objectives of this thesis were (1) to study preference or aversion or low or high appetence for Gly and L-amino acids in weaned pigs under a fed-status (*in vivo* trials – Chapter 1), (2) to demonstrate that pigs are able to detect and identify amino acids as umami tastants through the heterodimer pT1r1/pT1r3 (*in vitro* trials – Chapter 2), (3) to compare the *in vivo* data to those recorded by the *in vitro* system and (4) to study the expression of the pig umami taste receptor in taste and non-taste tissues from the gastrointestinal tract by age, sex and dietary protein content effects.

Pig preference and appetence for umami tastants under fed-status

Double choice (DOCH) tests are a useful methodology to study gustatory responses, preferences and appetence for nutrients and feed palatability in piglets (Kyriazakis, et al., 1990; Glaser et al., 2000; Tinti et al., 2000; Morgan et al., 2003; Ettle and Roth, 2004; Ettle and Roth, 2005; Roth et al., 2006; Solà-Oriol, 2008). We developed a very short DOCH test based on pigs' learning capacity for DOCH testing. It was the first time that gustatory responses by amino acid solutions were studied in pigs continuously fed "ad libitum". Also, the new model used a methodology for double-choice testing without a need for prior adaptation to social isolation keeping pigs in pairs during training and testing sessions. We studied pig preference, appetence and sensitivity for several amino acids using this methodology (Chapter 1). The duration of the test (2 min.), the quantity offered (around 250ml) and the number of test sessions per day (only 2 with an interval of 4h) were critical points to prevent cephalic responses and postingestional effects that could interfere with our results (Glaser et al., 2000; Tinti et al., 2000). According to Birch (1999), there is an innate predisposition to prefer or reject basic tastes, although those may be altered via experience with food and eating.

The umami taste is the only one of the five basic tastes that indicates the presence of amino acids, peptides and related structures (Bachmanov and Beauchamp, 2007; Roura and Tedo, 2009). Electrophysiological studies have shown that known umami tastants in humans are also a strong and positive taste stimulus for the pig, suggesting that umami tastants such as monosodium glutamate (MSG) and other L-AA may promote feed intake (Danilova et al., 1999). Studies of *in vivo* gustatory responses by L-AA in fasted pigs confirmed the electrophysiological recordings (Tinti et al., 2000). Similarly our *in vivo* data (Chapter 1) showed that potential umami tastants like MSG, L-Glu and L-Gln were highly preferred and very appetent to pigs under a fed-status whereas their preference and appetence was clearly low for essential amino acids such as L-Trp, especially at the highest tested concentration (500mM). We also reported that females showed a weak preference for 500mM L-Lys solution whereas males clearly rejected it. Owen et al. (1994) described that gilts chose a diet higher in lysine than barrows, probably because gilts require higher lysine content than barrows to maximize their growth or females may have a higher rejection threshold for this amino acid than males. Our results suggested that pigs may be able to recognize amino acids based on taste cues. Pigs seemed to be able to differentiate among different concentrations of essential amino acids (EAA). Furthermore they may have an innate

rejection response to dietary characteristics, such as an amino acid imbalance, that could trigger a undesirable postingestional effect. The postingestional consequences may determine consumption or avoidance of diets containing amino acid imbalances or deficiencies (Gietzen, 1993; Koehnle et al., 2003; Ettle and Roth, 2004). Nakamura et al. (2008) hypothesized that the preference for umami tastants is linked to the body requirements to metabolise ingested dietary protein. Umami tastants stimulate voluntary food intake and have positive postingestive effects through activation of several forebrain regions that have important roles in ingestive behaviour (Tsurugizawa et al., 2008). For example, in humans the addition of MSG and IMP in a first course may increase energy intake in a second course (Luscombe-Marsh et al., 2009) suggesting that these tastants have a positive postingestive effect that stimulates food intake.

There are several amino acid-sensing receptors that belong to the G-protein coupled-receptor family (GPCRs) like metabotropic Glutamate receptors (mGlu), Calcium receptors (CaR), T1R1/T1R3 heterodimer and G-protein coupled-receptor family C group 6 member A (GPRC6A) (Conigrave and Hampson, 2006; Wellendorph and Bräuner-Osborne, 2009). The *in vitro* identification and characterization of new ligands for GPCRs ideally requires the use of high throughput screening assays (HTS) based on cell reporter systems. These assays analyse the ligand-receptor binding interaction, normally through the measurement of second messenger signals that are detected by changes in a reporter gene expression, or in its transcription, and finally are determined by fluorescent dyes. Some of these *in vitro* systems showed that the mGlu receptors were monogamous for L-Glu (Frauli et al., 2006) although its ligand affinity was lower than for T1R1/T1R3 taste receptor (Raliou et al., 2009). The CaR, T1R1/T1R3 and GPRCA6 were promiscuous by nature and responded to a broad range of L-AA or divalent cations (Wellendorph et al., 2009). According to Wellendorph et al. (2009), these promiscuous amino acid receptors have a preference for a subset of classes of amino acids, potentially to cover responses to all of the 20 proteinogenic L-AA. For example, *in vitro* studies showed that CaR had the most potent response with aromatic amino acids like L-Phe and L-Trp and the least with acidic (L-Glu, L-Asp) and basic (L-Lys, L-Arg) amino acids (Conigrave et al., 2000). The T1R1/T1R3, on the other hand, responds highly to L-Glu and L-Asp and barely to aromatic amino acids (Li et al., 2002; Nelson et al., 2002). The heterodimer T1R1/T1R3 has been described in humans and laboratory rodents as the umami taste receptor for L-AA which response is enhanced by purinic ribonucleotides like IMP (Chaudhari et al., 2000; Li et al., 2002; Nelson et al., 2002; Conigrave and Hampson, 2006).

The sequences of the porcine umami taste receptor (pT*as*1r1 and pT*as*1r3 genes) were found in the tongue circumvallate papillae of pigs (Roura et al., 2008). It has higher homologies in its nucleotide and amino acid sequences with carnivores (i.e. felines and canines) than with other omnivores such as mice and humans (Humprey et al., 2009). Therefore, mouse and human may not be a good model for the pig umami sensing. According to García-Bailo et al. (2008), genetic variation in taste influences food selection. Further to that characterization, the pT*as*1r1 and pT*as*1r3 gene sequences were used to construct a stable cell reporter system that expresses both genes (Chapter 2). This *in vitro* system was used as the screening tool to identify and quantify the responses of potential porcine umami ligands, such as Gly and L-AA. The amino acid affinities to the heterodimer differ between mice, human and pig. In mice, the T1r1/T1r3 taste receptor responds broadly to all L-AA and Gly, except to aromatic amino acids, and the responses are enhanced by IMP (Nelson et al., 2002). In humans, this receptor was only very sensitive to L-Glu and L-Asp and their responses were also enhanced by nucleotides (Li et al., 2002). In pigs, the T1r1/T1r3 highly responds to MSG (umami taste by excellence- Ikeda, 1909), L-Glu, L-Gln, L-Asn, L-Ala and even Gly and has the least response with aromatic and sulphur amino acids (Chapter 2). The porcine umami taste receptor response normally increased as the dose of their ligands increased. We hypothesize that pT1r1/pT1r3 may also enhance its response to amino acids when nucleotides are added but further studies should be performed.

We also found that the pig preferences and appetence responses for some non-essential (NEAA: Gly, L-Ala, L-Gln, L-Glu and MSG) and essential amino acids (EAA: D,L-Met, L-Lys ,L-Thr and L-Trp) were related to their *in vitro* responses at 50mM (Chapter 2). Pig preferences and appetencies for the umami tastants (NEAA) were positively correlated with the same NEAA responses in our cell reporter system. The pig heterodimer was highly stimulated by MSG, L-Glu and L-Gln and those were highly preferred and consumed by ad libitum fed pigs and its response increased with concentration. Therefore, we may consider umami as a pleasant taste in pigs that may contribute to recognize sources of amino acids and peptides. We hypothesize that umami tastants may stimulate voluntary feed intake, especially in critical periods such as weaning.

Umami L-Glu and L-Gln tastants in pigs have several physiological and metabolic roles in the body (Bellisle, 1999). Both are NEAA, but L-Gln has been considered to be a conditional EAA in stressful conditions. The L-Gln supports the metabolic requirements of the intestinal mucosa when glucose depletion is severe and/or when it is damaged by starvation (Kim et al., 2007). This situation is common in weanling

pigs where depletion of intestinal nutrients due to low feed intake causes morphological and functional changes in the small intestine reducing nutrient digestion and absorption (McCracken et al., 1999; King and Pluske, 2003). The L-Glu and L-Gln are also present at high concentrations in piglet diets and at low levels in the blood of weaned pigs (Ayondrinde et al., 1995a, b). They are extensively oxidized in the small intestine epithelial cells and this oxidative capacity converges with a high energy demand of the intestinal epithelium due to its rapid renewal (Blachier et al., 2009). On the other hand, L-Glu modulates gastric secretions and motility (Uneyama et al., 2006; San Gabriel et al., 2007; Nakamura et al., 2008) .Therefore, these pig umami tastants are also nutrients of the gut mucosa that contribute to the development and construction of the intestinal epithelium and ameliorate digestive processes.

The age, sex and dietary protein effects in pT*as*1r1 and pT*as*r3 gene expression

Pigs perceive amino acids through the umami heterodimer transmembrane receptor T1R1/T1R3 system located in the taste bud cells (Chapter 2). The L-AA umami tastants are highly preferred by pigs (Chapter 1). The pT*as*1r1/pT*as*1r3 genes are expressed in the tongue what shows their implication in the oral nutrient sensing mechanism. Research relevant to other mammals have described the heterodimer expression located in taste buds of the tongue and soft palate (Hoon et al., 1999) but also in different parts of the gastrointestinal (GI) tract (Dyer et al., 2005; Bezençon et al., 2007). The T1R1/T1R3 promiscuity in relevant tissues allows this receptor to play a role in both oral but also GI and systemic nutrient-sensing and in several physiological processes as well (Rozegurt and Sternini, 2007; Sternini et al., 2008; Sbarbati et al., 2009). As it was mentioned before, the CaR is also a promiscuous amino acid-sensing receptor that highly responds to aromatic amino acids (Conigrave et al., 2000). This receptor was also found in several tissues of the GI tract (such as stomach, small and large intestine, liver, pancreas and gastric and enteric nerves with an important role in digestion and absorption of amino acids (Conigrave and Brown, 2006). Our results showed that the porcine T1r1/T1r3 umami receptor was also present in taste and non-taste tissues from the GI tract (Chapter 3) confirming the results from Kiuchi et al. (2006) that found the porcine pT*as*1r3 gene being expressed in the same tissues. The presence of the umami receptor in taste and non-taste porcine tissues suggests its implications as a sensor of sources of amino acids and peptides or other related structures in other parts of the GI tract. Further research should be granted in that area.

The impact of amino acids on gene expression has become an important area of research (Bruhat et al., 1999; Jefferson and Kimball, 2001; Averous et al., 2003). Mammals must adjust several physiological functions involved in the adaptation to amino acid availability by regulating the expression of numerous genes (Averous et al., 2003). Depletion of some amino acids due to deficiency of any or several essential amino acids, a dietary imbalance of amino acids or an insufficient intake of protein can induce gene expression changes (Bruhat et al., 1999; Jefferson and Scott, 2001). In piglets, gene expression is altered in small intestine by weaning and dietary glutamine supplementation (Wang et al., 2008). The results presented here represent the first time that the pTas1r1 and pTas1r3 gene expressions have been studied in taste and non-taste tissues in pigs as affected by age, sex and dietary crude protein content. Several studies have reported a different taste perception for nutrient solutions depending on age, sex and diet but have failed to show changes in taste receptor expression (Tordoff, 2007; Wardwell et al., 2009).

In humans, taste perception normally decreases with age and women have lower recognition thresholds than men (Wardwell et al., 2009). Also, individuals who preferred a high concentration MSG solution had a lower nutritional/protein status than those who preferred low concentration MSG solution (Murphy, 1987). Our results showed that the porcine umami taste receptor was fully developed at birth in both taste and non-taste tissues and its expression may change depending on the tissue (tongue and stomach seemed to have higher levels of expression than small intestine and liver), sex (males showed higher levels of expression after weaning than females), age (weaning caused changes in umami taste gene expression) and dietary protein content (a low crude protein diet upregulate the expression of the pig umami taste receptor).

The pTas1r3 gene expression was normally higher than that of pTas1r1. The umami heterodimer (T1R1/T1R3) share the T1R3 subunit with the sweet heterodimer (T1R2/T1R3) (Li et al., 2002; Nelson et al., 2002) and higher levels of expression of pT1r3 than pT1r1 may suggest the presence of sweet taste receptors in these analysed samples or its function as a taste receptor alone (Damak et al., 2003; Zhao et al., 2003). In our pigs, we found that the highest expression of both genes was in the stomach (fundic region), followed by the tongue, the small intestine and finally the liver. The expression in the pig tongue is similar in fungiform and circumvallate papillae for both genes that it is opposite to data from other mammals reporting that T1R1 had a higher expression in fungiform than in circumvallate papillae (Bachmanov and Beauchamp, 2007). Sex cause a high variability in the pTas1r1 and pTas1r3 gene expressions and thus it was included as a main effect in the generalized

estimating equation model. In general we found that males had higher levels of expression than females after weaning. However, further studies with a higher number of pigs are needed to confirm sex effect on the expression of the porcine umami dimer.

The most important changes of expression levels occurred in those organs responsible for digestion and absorption of amino acids, such as the stomach, duodenum and jejunum. According to our results, both genes seemed upregulated in the small intestine due to insufficient protein intake (48h after weaning) or low dietary crude protein content (20d after weaning with the LCP diet), thus they increased their expression under situations of high amino acid requirements and low luminal levels in the gut, especially after villous atrophy. Postweaning recovery of the intestinal structure and function lead to a high demand of amino acids such as glutamine, glutamate, aspartate and arginine by enterocytes, most of them from the intestinal lumen content (Miller and Slade, 2003).

According to Young et al. (2009), taste perception molecules are expressed in nutrient detection regions of the proximal small intestine, consistent with a role of tasting. The T1R2, T1R3, TRPM5 and Gα-gustducin expression were decreased in diabetic subjects with elevated blood glucose concentration. The T1R2 expression was decreased by luminal glucose in mice jejunum, indicating that intestinal taste receptor signaling was under dynamic metabolic and luminal control. On the other hand, Mace et al. (2009) demonstrated in rat jejunum that the sweet and umami taste receptors were coordinated for nutrient absorption. The physiological significance of coordinated regulation seems to be the control of energy supply, like glucose and non-essential amino acids (L-Glu and L-Asp) by enterocytes. Therefore, the umami taste receptor has a role in nutrient absorption processes.

The detection of amino acids starts in the tongue by this porcine umami taste receptor and continue in non-taste tissues of the GI tract. The upregulation of the dimer pT1r1/pT1r3, might be due to tBirch L.L. 1999. Development of food preferences.

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Conclusions

Conclusions >>>>>>>>

1 > The double-choice (DOCH) model maintaining pigs in pairs developed in this work avoids a previous adaptation period to social isolation, allows reducing training time (4 to 2 days) and test duration (2 minutes). The quantity of test solutions offered (approximately 250g) and the interval between test sessions (9am and 12pm) seems to prevent postingestional effects in the behavioural response.

2 > This model demonstrates that weaned pigs fed ad libitum were able to discriminate solutions of amino acids except when those were offered at 0.5mM.

3 > Pigs show high preference and appetence for potential umami tastants such as L-Gln, L-Glu and MSG that increase with concentration and rejection or aversion for essential amino acids such as D,L-Met, L-Lys, L-Thr and L-Trp at high concentrations (500mM).

4 > The *in vitro* system used in our work showed significant responses to non-essential amino acids, such as L-Glu, MSG, L-Gln, L-Asn, L-Ala and Gly in all tested concentrations, what suggests that this receptor is tuned to detect molecules derived from protein sources.

5 > The highest responses were observed with L-Glu, MSG, L-Gln, L-Asn and L-Ala, which are clearly umami tastans in pigs.

6 > The *in vivo* data was significantly correlated to the *in vitro* results meaning that the L-amino acids stimulating our cell reporter system were highly preferred by pigs. This cell reporter system can be used as a tool to identify other umami tastants pleasant pigs.

7 > The porcine umami taste receptor (T1r1/T1r3) is expressed in taste (tongue circumvallate and fungiform papillae) and non-taste tissues from the gastrointestinal tract (stomach, small intestine and liver) and its expression may change mainly in the small intestine with age, dietary crude protein content and sex.

8 > This receptor is fully developed at birth and the pT*as*1r1 and pT*as*1r3 genes may respond by increasing their expression at weaning.

9 > Both genes are also upregulated with low dietary protein feeds that presumably do not provide enough intestinal nutrients for villous construction and development after weaning.

10 > The pTasr1/pTasr3 gene expression is higher in males compared to females except in weaned pigs fed with low dietary crude protein content.

In summary, this work demonstrates that pigs sense some amino acids as umami tastants that generate a pleasant stimulus. The umami tastants are sensed through the heterodimer receptor pT1r1/pT1r3, which is expressed in taste buds in the tongue and in non-taste tissues along the gastrointestinal tract. Changes in pT*as*1r1/pT*as*1r3 gene expression may reflect the nutritional status of the animal and a better understanding of the mechanism will help to develop new strategies for improving the adaptation of piglets to the postweaning period such as the use of umami ligands to stimulate their voluntary feed intake.

Annex

Author Education and Training
List of Publications

Annex >>>>>>>>

Author Education and Training

Maria Gemma Tedó was born in 1975, in Sabadell, Barcelona, Spain. She finished her degree at the School of Veterinary Science of the Autonomous University of Barcelona (UAB) in 1999. She continued her studies following the animal production training plan at the School of Veterinary Science of the UAB and obtained her master degree in Animal Science in 2005. She combined her studies with her job as a nutritionist in Técnica Ganadera, S.L. and currently as a pig experimental unit manager in LUCTA, S.A. She has been developing part of this work at the experimental unit of LUCTA, S.A. and IRTA facilities (Mas de Bover-Reus), at the Biology faculty of the University of Barcelona (Celltec-UB) and at the Animal Science department of the University of California, Davis (UCDavis). This work was the basis of the current thesis submitted to obtain a PhD in Animal Science at the School of Veterinary Science of the UAB in 2009.

List of publications

Scientific publications

Tedó, G.; Larrosa, E.; Pifarré, M., Duarte, G., Benech, A, Cal, L., Manteca, X. and Rodas E. *Play behaviour in Corriedale lambs before weaning.* Congreso Iberoamericano de Fisiología Veterinaria, March 2000, Foz d'Iguaçú, Brasil.

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Scientific projects

Project: CDTI 20090124 "Cultivos celulares para la búsqueda in vitro de entidades químicas saborizantes para porcino". Main researcher: Dr. Eugeni Roura. Institution: LUCTA, S.A.