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Gut Commensal *E.coli* Proteins Activate Host Satiety Pathways Following Nutrient-Induced Bacterial Growth

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SUMMARY

The composition of gut microbiota has been associated with host metabolic phenotypes, but it is not known if gut bacteria may influence host appetite. Here we show that regular nutrient provision stabilizes exponential growth of *E.coli,* with the stationary phase occurring 20 min after nutrients supply accompanied by bacterial proteome changes, suggesting involvement of bacterial proteins in host satiety. Indeed, intestinal infusions of *E.coli* stationary phase proteins increased plasma PYY, their intraperitoneal injections suppressed acutely food intake and activated c-Fos in hypothalamic POMC neurons, while their repeated administrations reduced meal size. ClpB, a bacterial protein mimetic of α-MSH, was up-regulated in the *E.coli* stationary phase, was detected in plasma proportional to ClpB DNA in faeces and stimulated firing rate of hypothalamic POMC neurons. Thus, these data show that bacterial proteins produced after nutrient-induced *E.coli* growth may signal meal termination. Furthermore, continuous exposure to *E.coli* proteins may influence long-term meal pattern.

Key words: Energy balance; gut-brain axis; gut hormones; proteomics; obesity; eating disorders.

INTRODUCTION

The composition of gut microbiota has been associated with host metabolic phenotypes (Ley et al., 2006) and transfer of 'obese' microbiota can induce adiposity (Turnbaugh et al., 2006) and hyperphagia (Vijay-Kumar et al., 2010), suggesting that gut microbiota may influence host feeding behavior. Although the mechanisms underlying effects of gut bacteria on host appetite are unknown, it is likely that they may use the host molecular pathways.

The current model of appetite control involves gut-derived hunger and satiety hormones signaling to brain circuitries regulating homeostatic and hedonic aspects of feeding (Berthoud, 2011; Murphy and Bloom, 2006). Prominent amongst these are the anorexigenic and orexigenic pathways originating in the hypothalamic arcuate nucleus (ARC) such as the proopiomelanocortin (POMC) and neuropeptide Y (NPY)/ agouti-related protein (AgRP) neurons, which are relayed to the paraventricular nucleus (PVN) (Cowley et al., 1999; Garfield et al., 2015; Shi et al., 2013). The ARC and PVN pathways also converge in the lateral parabrachial nucleus, which sends anorexigenic calcitonin gene-related peptide (CGRP) projections to the central amygdala (CeA) (Carter et al., 2013). The CeA, among other forebrain areas, was shown to integrate homeostatic and motivational aspects of feeding and also receives a sensory input from the brainstem (Areias and Prada, 2015; Becskei et al., 2007; Morris and Dolan, 2001).

Putative mechanisms linking gut microbiota with the host control of food intake may involve energy harvesting activities of gut bacteria (Turnbaugh et al., 2006) or their production of neuroactive transmitters and metabolites (Dinan et al., 2015; Forsythe and Kunze, 2013). Another possibility, explored in this study, is that bacterial proteins may act directly on appetite-controlling pathways locally in the gut or via the circulation. In fact, several bacterial proteins have been shown to display sequence homology with peptide hormones (Fetissov et

al., 2008), and we have recently identified that the caseinolytic protease (Clp) B, produced by *Escherichia coli* (*E.coli*), is an antigen-mimetic of α-melanocyte-stimulating hormone (α-MSH) (Tennoune et al., 2014). α-MSH is a POMC-derived neuropeptide playing a key role in signaling satiation by activation of the melanocortin receptors 4 (MC4R) (Cone, 2005). Although MC4R-mediated α-MSH anorexigenic effects have been mainly ascribed to its central sites of actions (Mul et al., 2013), a recent study shows that activation of the MC4R in the gut enteroendocrine cells stimulates release of the satietogenic hormones glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (Panaro et al., 2014). Hence, local gut signaling by microbiota-derived α-MSH-like molecules to the enteroendocrine cells is possible (Manning and Batterham, 2014).

Most studies linking nutrition and microbiota have so far focused on the bacterial biodiversity (Parks et al., 2013), but little is known about how nutrient-induced bacterial growth may affect host metabolism. In fact, the dynamics of bacterial growth depend on nutrient supply, implying that regular daily meals should trigger the growth of gut bacteria. After a single provision of nutrients to cultured bacteria long-lasting exponential (Exp) and stationary (Stat) growth phases are observed which differ in protein expressions (Wick et al., 2001). It is, hence, possible that during the different growth phases induced by regular nutrient supply, gut bacteria will synthetize proteins that may differentially influence host appetite acting via intestinal and/or systemic routes.

To test this hypothesis, we studied growth dynamics of *E.coli* K12*,* a model organism of commensal strains of gut *E.coli* bacteria, exposed to regular nutrient supply, modelling two daily meals. Using proteomic approaches we compared proteins extracted from *E.coli* during the established pattern of alternations of the Exp and Stat growth phases and analysed the identified proteins for their relevance to energy metabolism. ClpB, a bacterial protein mimetic of α-MSH, was used as a marker of *E.coli* proteins which can be involved in signaling satiety.

We have developed a ClpB immunoassay, in order to determine if ClpB production and plasma concentrations may depend on the bacterial growth and intestinal delivery of nutrients. Pursuing the possibility that gut bacterial proteins may directly influence peripheral and central pathways involved in appetite control, we examined in mice and rats the effects of *E.coli* proteins from the Exp or Stat growth phases on food intake and meal pattern, plasma GLP-1 and PYY and activations of some key appetite-regulating neurons in the brain such as in the ARC and CeA.

RESULTS

E.coli Growth Dynamics in Vitro After Regular Nutrient Provision and Proteomic Analysis

A regular, each 12 h provision of Müeller-Hinton (MH) nutritional medium to cultured *E.coli* increased bacterial biomass and shortened the Exp growth phase (Fig. 1A,B). As such, after the 5^{th} MH medium supply, the Exp growth phase lasted for 20 min, and it did not further change after the following nutrient provisions with the same (Δ0.3) relative increase in OD, reflecting an identical bacterial growth (Fig. 2A-C). According to the McFarland standards, an increase of 0.3 OD corresponds to an increment of 10^8-10^9 of bacteria.

Because the growth dynamics of regularly-fed bacteria can be associated with the host prandial and postprandial phases, we have compared the proteomes of *E.coli* extracted in the middle of the Exp phase, i.e. 10 min after nutrient provision, and in the Stat phase 2 h later, a time normally characterized by a feeling of satiety in the host (Fig. 1C). Two-dimensional polyacrylamide gel electrophoresis was performed separately on membrane and cytoplasmic fractions (Fig. 1D,E). The total number of detected protein spots was 2895 (1367 membrane and 1528 cytoplasmic). Comparative analysis revealed 20 differentially expressed (by at least

1.5 fold) membrane proteins (Suppl. Fig. 1A,B). Among them, 17 proteins showed increased expression in the Exp phase and of these, 15 were identified by mass spectrometry (Suppl. Fig. 1C). Contrary to the membrane proteins, 19 from 20 differentially expressed cytoplasmic proteins showed increased levels during the Stat phase (Suppl. Fig. 2C). Only flagellin had higher expression in the Exp phase. The majority of identified proteins were implicated in either anabolic or catabolic processes (Suppl. Tables 1,2) showing an overall mixed metabolic profile in both growth phases, as summarized in Fig. 1F. Thus, these data show that the proteomes of regularly fed *E.coli* are qualitatively different between growth phases, although their metabolic profiles are not clearly distinguishable.

After the 9th nutrient provision, total bacterial proteins were extracted in the Exp and Stat phases displaying concentrations of 0.088 mg/ml and 0.15 mg/ml, respectively, and were used in the ATP production assay, tested for ClpB levels and used for intracolonic infusions and intraperitoneal (I.P.) injections.

ATP Production Capacity by *E.coli* Proteins in Vitro is Similar for Both Growth Phases

To verify if proteome changes between growth phases may influence bacterial energy extraction capacities, the adenosine-5'-triphosphate (ATP) production by *E.coli* K12 proteins from the Exp and Stat phases was tested *in vitro.* We found that proteins from both growth phases were able to increase ATP production from different energy sources (Fig. 1G). The ATP concentrations were higher, when a protein-containing mixed energy source, such as the MH medium was used as compared to a sucrose solution. The ATP production increased dose-dependently with concentrations of bacterial proteins. However, no significant differences were found between ATP-producing effects of proteins from the Exp or Stat phases (Fig. 1G). These results confirm that bacterial proteins may continue to catalyze ATP production after

bacterial lysis suggesting that nutrient-induced bacterial growth may contribute to increased ATP production in the gut. Changes in whole-body ATP content can be relevant to appetite control via regulation of the activity of adenosine-5'-monophosphate-activated protein kinase, resulting in increased food intake, when ATP levels are low and *vice versa* (Dzamko and Steinberg, 2009). Furthermore, increased intraluminal ATP production may contribute to the digestive process via gut relaxation (Glasgow et al., 1998).

Increased *E.coli* ClpB Production in the Stat Growth Phase

E.coli ClpB is a conformational protein mimetic of α-MSH, and may be potentially involved in *E.coli* effects on host feeding. We developed and validated an enzyme-linked immunosorbent assay (ELISA) for detection of *E.coli* ClpB (for details see Experimental procedures and Suppl. Fig. 3). To investigate whether ClpB production is different between two growth phases, we used Western Blot and ELISA. ClpB-corresponding, 96 KDa bands was detected in all proteins preparations, with an increased mean level during the Stat phase (Fig. 1H,I). These changes were further confirmed using the ClpB ELISA, showing almost doubling of ClpB concentrations in the Stat phase (Fig. 1J).

In vivo Nutrient-Induced Bacterial Growth & Effects of *E.coli* Proteins in the Gut to Stimulate GLP-1 and PYY Plasma Release

To verify if our *in vitro* model of regular nutrient-induced *E.coli* growth is relevant to gut bacterial growth dynamics *in vivo*, MH medium or water were infused into the colon of anaesthetized rats. We found that instillations of the MH medium, but not water, induced bacterial proliferation in the gut with the Exp growth phase lasting for 20 min (Fig. 2A), consistent with our *in vitro* data. To see if the bacterial growth in the gut may be accompanied

by increased plasma ClpB, its concentrations were measured in the portal vein before and after colonic infusions. ClpB was readably detected in rat plasma but no significant differences were found 30 or 60 min after MH infusion (Fig. 2B).

Nevertheless, plasma ClpB concentration correlated positively with ClpB DNA content in rat faeces (Fig. 2C); presence of such correlations were confirmed by an independent study in rats (data not shown). Furthermore, plasma ClpB levels were increased in mice after 3 weeks of gavage with wild type but not with ClpB mutant *E.coli* (Suppl. Fig. 3F). These data indicate that plasmatic ClpB depends on number of ClpB-expressing bacteria in the gut but it cannot be a short-term signal of satiation to the brain.

Next, we determined if growth-dependent changes of *E.coli* proteomes may influence host appetite control locally in the gut via release of satiety hormones. In a separate experiment, anaesthetized rats received 20 min colonic infusions of *E.coli* proteins from the Exp or Stat phases, both at 0.1 mg/kg. The concentrations of ClpB in the colonic mucosa measured 20 min after the infusion were higher in rats receiving the Stat phase proteins (Fig. 2D) but it was not affected in plasma (Fig. 2E). The colonic infusions of *E.coli* proteins from the Exp, but not the Stat phase, stimulated plasma levels of GLP-1 (Fig. 2F) and, in contrast, increased plasma levels of PYY were detected after infusion of proteins from the Stat, but not the Exp, phase (Fig. 2G). Thus, *E.coli* proteins produced during nutrient-induced bacterial growth in the gut may influence short-term appetite control via stimulation of gut satiety hormone release.

Food Intake and Hypothalamic and Amygdala c-Fos Activation After Acute *E.coli* Protein Administration in Rats

Because *E.coli*-derived ClpB was present in plasma of rats and mice, it is possible that plasmatic *E.coli*-derived proteins might influence long-term appetite control via a systemic route. Testing this possibility in overnight fasted rats, we found that a single I.P. administration (0.1 mg/kg) of the Stat phase membrane fraction of *E.coli* proteins, decreased 1 h- and 2 h-food intake during refeeding as compared with the control group (Fig. 3A). In contrast, administration of the cytoplasmic fraction of the Exp phase *E.coli* proteins increased 4 h food intake (Fig. 3B). Because both cell protein fractions are simultaneously present and in order to see if *E.coli* proteome may influence spontaneous food intake, free feeding rats were injected before the onset of the dark phase with total bacterial proteins (0.1 mg/kg, I.P.). Food intake was measured for 2 h after injections, and was followed by the immunohistochemical analysis of c-Fos expression in brain. We found that rats injected with bacterial proteins from the Stat phase ate less than controls, while food intake was not significantly affected by injections of bacterial proteins from the Exp phase (Fig. 3C).

An increased number of c-Fos-positive cells was found in the ARC of rats receiving Stat phase proteins (Fig. 4D). The majority of c-Fos expressing cells in the ARC contained β-endorphin (Controls, 71.31 ± 12.81%, *E.coli* exp. phase, 73.56 ± 10.45% , *E.coli* stat. phase, 80.50 ± 9.68%, ANOVA p=0.36), i.e. were identified as anorexigenic POMC neurons (Fig. 4A-C). Although the total numbers of β-endorphin-positive cells were not significantly different among the groups (Controls, 54.82 ± 10.67 cells, *E.coli* exp. phase, 66.03 ± 11.43 cells, *E.coli* stat. phase, 66.03 ± 5.06 cells, ANOVA p=0.09), the relative number of c-Fos activated β-endorphin neurons was increased in rats receiving Stat phase proteins (Fig. 4E). Furthermore, the number of c-Fos activated β-endorphin neurons correlated inversely with food intake (Fig. 4F).

C-Fos expressing cells were also analyzed in the ARC neighboring ventromedial nucleus (VMN, Fig. 4G-I) showing their increased number in rats receiving bacterial proteins from the Stat phase (Fig. 4J).

Similarly, in the CeA, the number of c-Fos-positive neurons was increased in rats injected with the Stat phase proteins (Fig. 4O) and correlated inversely with food intake (Fig. 4P). We used double-immunostaining to see if c-Fos activated CeA neurons were located in the terminal field of CGRP-positive fibers (Fig. 4K-M). A confocal microscopy revealed that c-Fos expressing neurons in the CeA were often surrounded by CGRP fibers (Fig. 4N), confirming that they were located in the terminal field of anorexigenic projections from the parabrachial nucleus. Thus, acute systemic increase in *E.coli* proteins from the Stat phase was associated with decreased food intake and activation of anorexigenic neurons including both accessible to circulating factors such as in the ARC and in the brain-downstream nuclei such as CeA.

Feeding Pattern and Hypothalamic Neuropeptides After Chronic *E.coli* Protein Injections in Mice

To determine the potential long-term effects of systemic presence of *E.coli* proteins on body weight, food intake and feeding pattern, two daily injections of *E.coli* total proteins (0.1 mg/kg, I.P.) were administered for one week to free feeding mice. The first day after injections was characterized by significantly lower body weight and food intake in mice receiving bacterial proteins from the Stat but not the Exp phase (Fig. 5A,B). Although daily meal size was not significantly different among the groups, its decrease relative to the day before injection was observed after one week in mice receiving Stat phase proteins as compared to controls (Fig. 5C). In contrast, meal frequency in these mice showed an increasing trend (Fig. 5D). Whereas total food intake among the 3 groups was not significantly different during the study period,

mice injected with the Exp phase proteins had increased food intake during the light (inactive) period (Fig. 5E), and decreased during the dark (active) period (Fig. 5F). In contrast, mice receiving the Stat phase proteins displayed lower food intake than controls in the dark period without any effect in the light period (Fig. 5E,F). While during the first day after injections mice receiving proteins from the Stat phase displayed increased satiety ratio (Fig. 5G), reflecting an increased duration of post-meal intervals relative to the amount of food eaten during preceding meals, the same group showed a tendency towards a decrease at the end of the study (Fig. 5H).

To get an insight into the molecular changes underlying altered feeding pattern observed after 6 days of bacterial protein injections, we analyzed hypothalamic mRNA expression levels of several neuropeptides involved in appetite control. We found that mice receiving the Stat phase proteins showed elevated precursor mRNA levels of anorexigenic brain-derived neurotrophic factor (BDNF) and of orexin as compared to controls, and of anorexigenic corticotropin-releasing hormone (CRH) as compared to mice injected with proteins from the Exp phase. The latter also showed elevated mRNA levels of BDNF but decreased mRNA levels of the precursor of orexigenic pyroglutamylated RFamide peptide (QRFP) (Suppl. Table 3). These data suggest that long-term systemic effects of *E.coli* proteins in mice may influence their meal pattern without affecting total energy balance via modulation of hypothalamic neuropeptide expression.

Electrophysiological Activation of Hypothalamic POMC Neurons by *E.coli*-Derived ClpB

To confirm that bacterial proteins present in the circulation may directly activate feeding-related brain circuitry, we used an electrophysiological approach. We looked if ClpB, an α-MSH mimetic protein and a marker of *E.coli* proteins up-regulated in the Stat phase, may

directly activate ARC POMC neurons. Brain slices from POMC-eGFP mice were examined using cell-attached patch-clamp electrophysiology (Fig. 6A,B). We found that, bath addition of ClpB (1 nM) increased action potential frequency of ~50 % of the tested ARC POMC neurons (n =7/13) by 229 ± 109 % (basal: 2.02 ± 0.78 Hz *vs.* ClpB: 3.82 ± 1.36 Hz, Fig. 6C,D). In general, POMC neurons did not fully return to their basal firing rate until at least 10 min after ClpB application (Fig. 6C,D).

DISCUSSION

Our study reveals that bacterial proteins may physiologically link gut *E.coli* to host control of appetite involving both short-term effects on satiation, associated with nutrient-induced bacterial growth, acting locally in the gut, as well as long-term regulation of feeding pattern associated with plasmatic changes of bacterial proteins that may activate central anorexigenic circuitries. The following main results support this conclusion: *1)* regular provision of nutrients stabilizes the Exp growth of *E.coli* lasting for 20 min both *in vitro* and *in vivo*; *2) E.coli* Stat growth phase was characterized by increased total bacterial protein content and a different proteome profile, including increased expression of ClpB, a bacterial protein mimetic of α-MSH; *3) E.coli* proteins dose-dependently stimulated *in vitro* ATP production; *4)* Plasma levels of ClpB did not change after nutrient-induced bacterial growth in the gut, but correlated with ClpB DNA in gut microbiota and was increased after chronic *E.coli* supplementation; *5)* Intestinal infusion of *E.coli* proteins from the Exp or Stat growth phases increased plasma GLP-1 or PYY levels, respectively; *6)* Acute I.P. administrations of *E.coli* proteins from the Stat phase decreased food intake and led to c-Fos activation in anorexigenic ARC and CeA neurons while their chronic I.P. injections reduced meal size without affecting total food intake and body weight, and finally *7)* ClpB stimulated firing rate of ARC POMC neurons *ex vivo*.

Regular Provision of Nutrients and Bacterial Growth

Among the wide variety of bacteria in the gastrointestinal tract, *E.coli* is the most abundant facultative anaerobe, justifying it as a model organism for commensal gut bacteria. Here, we show that during regular nutrient supply to cultured *E.coli* the growth dynamics of a rich bacterial population is characterized by an immediate Exp growth entering the Stat phase 20 min after nutrient supply. The growth cycle, which is apparently limited to only one division of bacteria, is then identically reproduced after the next provision of nutrients, suggesting that it can play a role of a pacemaker. A similar dynamics of bacterial growth in response to nutrient infusion was seen in the rat colon, supporting that our *in vitro* data can be relevant to *in vivo* situation, e.g. in humans taking regular meals, and that it is not limited to *E.coli*. The 10^8-10^9 increase of bacterial number remained stable after each new provision, suggesting that the corresponding stable production of the bacterial biomass in the gut, including proteins, may play a role in regulation of host feeding. Given that the average prandial phase in humans is similar to the duration of the Exp growth of regularly-fed bacteria, it is tempting to speculate that host satiety may be triggered by gut bacteria reaching the Stat phase 20 min after a contact with ingested nutrients. However, bacterial content in the gastrointestinal tract ranges from 10^3 in the stomach to 10^{12} in the colon. Moreover, about 2 h is necessary for the advancement of ingested nutrients through the stomach and small intestine, and the transit through the large intestine requires about 10 h. Because of such a delay of nutrient delivery to most gut bacteria, it is likely that beside the direct contact with the nutrient bolus, bacterial growth during the prandial phase might also be initiated by nutrients released into the gut lumen by the Pavlovian cephalic reflex to ingestion (Power and Schulkin, 2008).

Bacterial Protein Expression During Growth Phases and Intestinal Sensing

Using ClpB as a marker of *E.coli* proteins helped us to determine the putative action sites of bacterial proteins on host appetite pathways. ClpB is a chaperone protein present in both cytosolic and membrane *E.coli* compartments (Winkler et al., 2010); its increased expression may save bacteria from elevated protein aggregations in the Stat phase (Kwiatkowska et al., 2008). From the other hand, as an α-MSH mimetic, increased ClpB production may contribute to the activation of anorexigenic pathways after nutrient-induced *E.coli* proliferation. Although bacterial proteins are present in the intestinal mucosa (Haange et al., 2012), their passage across the gut barrier has not been reported. In theory, after spontaneous or induced bacterial lysis in the gut (Rice and Bayles, 2008), bacterial components may pass across the mucosal epithelial barrier by absorption in enterocytes and by paracellular diffusion. For example, lipopolysaccharide, which is released upon lysis of gram-negative bacteria, is naturally present in plasma of healthy humans and mice with higher basal levels after consuming high-fat meals (Cani et al., 2007). Here we show that bacterial ClpB is present in rat plasma with levels remaining stable following bacterial proliferation in the gut or after intestinal infusion of bacterial proteins. This indicates that plasmatic ClpB, and likely other bacterial proteins present in plasma, are not acutely influenced by nutrient-induced bacterial proliferation and, hence, their potential effects on the short-term satiety signaling should be limited to the gut.

Nevertheless, plasma ClpB concentrations correlated with ClpB DNA in gut microbiota, suggesting that the number of ClpB-producing bacteria, which should be at long-term relatively independent from its daily fluctuations of the nutrient-driven bacterial growth (Liang et al., 2015; Zarrinpar et al., 2014), may be the main factor responsible for the long-term maintenance of plasma ClpB levels. This conclusion is further supported by our data,

obtained upon validation of ClpB ELISA, showing increased plasma ClpB concentrations in mice chronically gavaged with *E.coli* but not in mice that received ClpB-mutant *E.coli* (Suppl. Fig. 3). Thus, gut bacterial proteins present in plasma such as ClpB, may act systemically potentially linking the composition of gut microbiota with the long-term control of host food intake.

Intestinal Effects of *E.coli* Proteins on Satiety Hormones

Next, we studied if *E.coli* proteins in the gut may stimulate the systemic release of gut satiety hormones such as GLP-1 and PYY (Adrian et al., 1985; Flint et al., 1998). In fact, both hormones are produced by the same or distinct enteroendocrine L-cells, present throughout the intestine and especially abundant in the colon (Eissele et al., 1992). These cells are directly and continuously exposed to gut bacteria and their fragments, including proteins. We found differential effects of *E.coli* proteins on GLP-1 and PYY release, with stimulation of GLP-1 levels by proteins from the Exp phase and of PYY levels from those of the Stat growth phase. These results point to some similarities between nutrient-induced bacterial growth and the known dynamics of meal-induced release of GLP-1 and PYY. In fact, as shown in humans, an acute peak of plasma GLP-1 occurs 15 min after an intragastric infusion of a liquid meal, while a longer-lasting elevated plasma PYY starts between 15-30 min after a meal (Edwards et al., 1999; Gerspach et al., 2011). A longer release of GLP-1 has been associated with fat intake (van der Klaauw et al., 2013). Thus, the growth dynamics of regularly-fed gut bacteria fits temporally into the dynamics of host GLP-1 and PYY release, suggesting an inductive role of gut bacteria, and specifically of *E.coli* proteins, in meal-induced signaling of intestinal satiety and meal termination. A differential effect of *E.coli* proteins from the Exp phase to stimulate GLP-1, may possibly reflect an incretin role of GLP-1 in glycemic control (Edwards et al., 1999).

Presence of functional MC4R in L-cells (Panaro et al., 2014) may possibly underlie activation of these cells by bacterial proteins containing α-MSH-mimetics such as ClpB.

Systemic Effects of *E.coli* Proteins on Food Intake and

Appetite-Regulating Brain Pathways

We showed here that administration of *E.coli* proteins in hungry or free-feeding rats and in free-feeding mice changed acutely their food intake depending on the growth phase of *E.coli*. Because the ATP production by Exp and Stat proteins was similar, such changes in food intake must involve other underlying mechanism than energy extracting capacities of bacterial proteins. Considering that plasma ClpB was not affected by intestinal infusion of nutrients and was stable over short-time, any systemic action of bacterial proteins on appetite should be interpreted as relevant to their long-term modulatory effects. Furthermore, because of the short duration of the Exp growth phase of regularly-fed bacteria, bacterial proteins upregulated during the long-lasting Stat phase should dominate in plasma under physiological situations. We found that *E.coli* Stat phase proteins produced generally acute anorexigenic responses in both hungry and free-feeding animals. However, after their chronic delivery the daily food intake was normalized. A progressive decrease of meal size in chronically-injected mice was accompanied by increased meal frequency, most likely as a compensatory mechanism to maintain total food intake. Accordingly, the pattern of mRNA expression of appetite-regulating neuropeptides in the hypothalamus showed a mixed response with activation of both anorexigenic and orexigenic pathways. Of note, both groups of mice receiving *E.coli* proteins showed a similar increase of BDNF mRNA, an anorexigenic pathway downstream of MC4R in the VMN (Xu et al., 2003). This pathway may underlie a decreased food intake during the dark phase observed in both groups. This effect was further

accentuated in mice injected with the Exp phase proteins showing lower levels of orexigenic QRFP (Chartrel et al., 2003) and NPY (Herzog, 2003). By contrast, mice that received Stat phase proteins displayed an enhanced anorexigenic profile with elevated levels of CRH mRNA, most likely implicating MC4R-expressing PVN neurons (Lu et al., 2003). These changes, combined with increased mRNA precursor expression of orexin A that stimulates meal frequency (Baird et al., 2009), may contribute to the altered meal pattern after 6 days of injections. Thus, these results support a role of systemically present *E.coli* proteins in regulation of meal pattern, without affecting long-term energy balance.

Consistent with our hypothesis that bacterial proteins produced during the Stat phase may activate some key central anorexigenic pathways, we found an increased c-Fos expression in the ARC POMC neurons as well as in the VMN. This nucleus has long been known as a satiety center and is interconnected with the ARC POMC neurons (Sternson et al., 2005). The obtained c-Fos pattern resembles that of a satietogenic response during food ingestion (Johnstone et al., 2006) or induced by satiety hormones such as PYY or pancreatic polypeptide (Batterham et al., 2002; Blevins et al., 2008; Challis et al., 2004; Lin et al., 2009). A relatively small number (~10%) of c-Fos-activated POMC neurons suggests that circulating *E.coli* proteins might have a modulatory effect on appetite control acting via this hypothalamic pathways. Although it was not feasible to determine c-Fos activation by the NPY/AgRP neurons, their contribution to signaling by bacterial proteins cannot be excluded; these neurons also express MC3R and MC4R (Mounien et al., 2005). Moreover, a stronger activation of c-Fos in the field of anorexigenic CGRP projections to the CeA (~40%) may signify a convergent down-stream action from ARC POMC and NPY/AgRP neurons, and possibly from other appetite-regulating brain areas, such as the nucleus of the solitary tract.

Finally, to determine if bacterial circulating proteins might have direct effect on appetite-regulating brain sites, we applied ClpB on hypothalamic slices which contain, among others, the ARC POMC neurons. Our results show that about 50% of the studied POMC neurons increase their action potential frequency, remaining activated for at least 10 min. The sustained effect of ClpB is consistent with the effect of α-MSH on POMC neurons expressing functional MC3R and MC4R (Smith et al., 2007). However, we do not know if ClpB was able to activate POMC neurons by direct binding to these receptors or indirectly via a local neuronal network. In either cases, our results point to a role of ClpB as a physiological activator of the hypothalamic anorexigenic pathway, somewhat similar to satietogenic PYY and leptin (Batterham et al., 2002; Cowley et al., 2001).

Taken together, these data support a role of systemically-present *E.coli* proteins in influencing host appetite via activation of brain anorexigenic pathways, possibly involving bacterial mimetic proteins of peptide hormones such as ClpB. The results also suggest that changes of microbiota composition, resulting in low or high abundance of *E.coli,* and possibly of other bacteria expressing the same proteins, e.g. from the *Enterobacteriaceae* family, may influence via a systemic route the meal pattern. Future studies should determine, if gut bacteria belonging to other families may also express proteins with appetite-modulating properties and clarify their possible involvement in the host metabolic phenotypes.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures are shown in Supplementary materials.

In vitro *E.coli* growth and proteomic analysis

E.coli K12 bacteria were cultured at 37°C in MH medium. *E.coli* protein extracts were resolved by isoelectric point using the IPGphor isoelectric focusing system (GE Healthcare). Molecular weight separation was done using Ettan Daltsix vertical electrophoresis system (GE Healthcare). Gels proteins were visualized by CBB G-250 staining (BIO-RAD) and analyzed using ImageMaster 2D Platinum 5.0 software (GE Healthcare). The protein spots of interest were excised using the Ettan Spot Picker (GE Healthcare), and automated in-gel digestion of proteins was performed on the Ettan Digester (GE Healthcare). Protein extracts were analyzed with a nano-LC1200 system coupled to a 6340 Ion Trap mass spectrometer equipped with a nanospray source and an HPLC-chip cube interface (Agilent Technologies). For protein identification, MS/MS peak lists were extracted and compared with the protein databases by using the MASCOT Daemon search engine 2.2.2 (Matrix Science).

ATP assay

Bacterial proteins from the Exp or Stat phases were added to the plate in duplicates with 3 concentrations (1, 10 and 25 µg/mL), adjusted with ATP assay buffer to the same volume and incubated with different nutrients solution including 15% sucrose or MH medium 2 h at 37°C. After the incubation, ATP was measured using an ATP assay kit according to the manufacturer's instructions (BioVision).

Development and validation of ClpB immunoassay

Design of the ClpB detection assay was based on several criteria, such as a specific and sensitive detection in a linear concentration range (Suppl. Fig. 3A). For this assay both polyclonal and monoclonal anti-ClpB antibodies were generated. To verify that ClpB plasma assay detects ClpB derived from gut bacteria, we used the ClpB ELISA to measure ClpB in plasma of mice which had been supplemented via intragastric gavage daily for 3 weeks with WT or with ΔClpB *E.coli*. Plasma samples were available from our previous study (Tennoune et al., 2014). We found that ClpB was normally present in mouse plasma including both controls and mice gavaged with a culture broth without bacteria. Importantly, ClpB plasma levels were increased in mice receiving WT *E.coli* but were unchanged in mice supplemented with ClpB-deficient *E.coli*, confirming the gut bacterial origin of plasmatic ClpB (Suppl. Fig. 3F).

Intestinal administrations of *E. coli* proteins in rats

Animal care and experimentation were in accordance with guidelines established by the National Institutes of Health, USA and complied with both French and European Community regulations (Official Journal of the European Community L 358, 18/12/1986). Female Sprague-Dawley rats, body weight 200-250 g (Janvier Labs, Genest-Saint-Isle, France) were kept in holding cages (3 rats per cage) in a fully equipped animal facility under regulated environmental conditions (22 ± 1 ˚C, on a 12 h light–dark cycle with lights on at 7:30 a.m.) for 1 week in order to acclimatize them to the housing conditions. Standard pelleted rodent chow (RM1 diet, SDS, UK) and drinking water were available *ad libitum*.

Experiment #1

Rats were anaesthetized by ketamine / xylazine solution and after a laparotomy, the colon was mobilized by placing 2 ligatures: 1st at the caecocolonic junction and the 2nd, 4 cm below. Colonic infusions and luminal content sampling were performed using a polypropylene catheter inserted into the ascending colon and fixed with the 1st ligature. 2 ml of MH medium or water were infused into the colon and immediately thereafter withdrawn for the measurement of OD. Blood samples were taken from the portal vein before and 30 and 60 min after the 1st infusion. Faecal samples were taken from the colon at the end of experiment for DNA extraction and PCR of ClpB.

Experiment #2

Rats were anaesthetized and the colon was mobilized as described above. *E.coli* proteins (0,1 µg/kg in 2 ml of PBS) were infused into colon once for 20 min. Blood samples were taken from the portal vein before and after infusions for assays of GLP-1, PYY and ClpB. Samples of colonic mucosa were taken for ClpB ELISA assay. GLP-1 and PYY assays were performed using a fluorescent enzyme immunoassay kit (Phoenix Pharmaceutical Inc., CA), according to the manufacturer's instructions.

Administrations of *E. coli* proteins in rats, food intake and brain c-Fos study

Male Wistar rats, were acclimatized to the housing conditions and were fed as described above. Two experiments including food restriction were performed in the same rats with 4 days interval. The 3rd experiment in free feeding rats involved their new series. The experimental design is presented in details in supplementary materials. Food intake was measured during 4h in first two experiments and 2h in the 3rd experiment after which rats

were anaesthetized by sodium pentobarbital (0.2 mg/kg, I.P.) and perfused for the immunohistochemical study of c-Fos expression in the brain. Positive cells were counted at x20 magnification from 6 consecutive sections.

Chronic administrations of *E. coli* proteins in mice

Two-month-old male C57Bl6 mice were placed in the BioDAQ mouse cages (Research Diets, Inc., New Brunswick, NJ) and divided into three groups (*n*=8), each receiving for one week two daily I.P. injections at 9:00 and at 18:30 of either: (i) PBS, (ii) bacterial proteins in Exp or (iii) in Stat phase (0,1 mg/Kg of body weight). Feeding data was analyzed using the BioDAQ data viewer 2.3.07 (Research Diets). For the meal pattern analysis, the inter-meal interval was set at 300 s. The satiety ratios were calculated as time (s) of post-meal interval divided by amount of food (g) consumed in the preceding meal. After the experiment, mice were killed by decapitation; the brain was removed and the hypothalamus dissected for the neuropeptide mRNA microarray.

Electrophysiological recordings

Brain slice were prepared from adult POMC-eGFP mice as previously described (Fioramonti et al., 2007) and incubated in oxygenated extracellular medium for a recovery period at least 60 min at RT. Cell-attached recordings were made using a Multiclamp 700B amplifier, digitized using the Digidata 1440A interface and acquired at 3 kHz using pClamp 10.3 software (Axon Instruments, Molecular Devices, Sunnyvale, CA). After a stable baseline was established, 1 nM of ClpB (Delphi Genetics) was perfused for 5-10 minutes. The POMC neurons' firing rate was measured over the last 3 min of the ClpB perfusion, 7-10 min after the perfusion and compared with the firing rate measured 3 min before the perfusion.

Statistical analysis

Data were analyzed and the graphs were plotted using the GraphPad Prism 5.02 (GraphPad Software Inc., San Diego, CA). Data are shown as means ± standard error of means (SEM), and for all test, $p < 0.05$ was considered statistically significant.

AUTHOR CONTRIBUTIONS

Study concept & experimental design: SOF. Microbiology experiments: JB, NT, JP. Proteomics: JB, NT, AG, PC. Animal and histology experiments: JB, NL, RL, MF, CG, JJ. PCR microarray design: DV. Electrophysiology: FL, XF. Data analysis: JB, SOF. Manuscript writing: SOF and JB. Project support: MPC, DV, ISE, LP, TH, PD. All authors edited or commented on the manuscript.

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Figure legends

Figure 1. Effects of repeated nutrient supply on *E.coli* growth *in vitro* and proteomic analysis. **A**,**B.** Dynamics of *E. coli* K12 bacterial growth during 9 regular provisions of MH medium. **C.** Proteins were extracted during the last growth cycle as indicated by arrows during the Exp phase (Exp, **a**) and the Stat phase (Stat, **b**). Representative images of Coomassie Brilliant Blue-stained 2-D gels of cytoplasmic proteins extracted from *E. coli* K12 in Exp (**D**) and in Stat (**E**) phases, MW, molecular weight. **F**. Number of *E.coli* proteins increased in each growth phase in relation to their catabolic or anabolic properties. **G.** Effects of bacterial proteins on ATP production *in vitro* from different food substrates. ClpB protein levels ±SEM in 2 growth phases was analyzed by Western Blot (**H**,**I**) and ELISA (**J**). OD, optical density. Student's *t*-test, **p*<0.05.

Figure 2. Effects of intestinal infusions of nutrients and *E.coli* proteins in rats. **A.** Bacterial growth dynamics after colonic instillations of MH medium, and (**B**) plasma ClpB before and during instillations. **C.** Detection of ClpB DNA in rat faeces and its correlation with plasma ClpB levels before instillations. Effects of colonic infusion of *E.coli* proteins from Exp and Stat growth phases on concentrations of ClpB in colonic mucosa (**D**) and plasma (**E**), and plasma concentrations of GLP-1 (**F**) and PYY (**G**), before (T_0) and 20 min after infusions (T_{20}). **D**, Student's *t*-test, **p*<0.05, **F**,**G**, ANOVA, *p*=0.02 and *p*= 0.0004, respectively, Tukey's post-test * *p*<0.05 and ****p*<0.001. All data are shown as mean±SEM.

Figure 3. Food intake after acute injections of *E.coli* proteins in rats. A,B. Food intake after I.P. injections of membrane (**A**) and cytoplasmic (**B**) fractions of *E.coli* proteins from Exp and Stat growth phases or PBS as a control (Contr) in rats during refeeding after overnight food restriction. **C.** 2h food intake in free-feeding rats injected with total *E.coli* proteins before the onset of the dark phase. **A,** ANOVA p=0.04, Tukey's post-test Contr. vs. Stat., *p<0.05, Student's t-test Contr. vs Stat. $^{\$}p$=0.01. **B,** Student's t-tests, Exp. vs. Contr. and vs. Stat. $^{\$}p$<0.05. **C**, ANOVA p=0.05, Tukey's post-test Contr. vs. Stat., *p<0.05 and Student's t-test Exp. vs. Stat. $^{\$}p$=0.04. All data are shown as mean±SEM.

Figure 4. Effects of *E.coli* proteins on c-Fos expression in the brain. Immunohistochemical detection of c-Fos (green) in the ARC (**A**-**C**), VMN **(G-I)** and CeA **(K-N)** 2 h after I.P. injections of Exp. and Stat. *E.coli* proteins in rats. Double staining with β-endorphin (β-end, red) in the ARC (**A**-**C**) and with CGRP in the CeA (red, **K**-**N**) including a confocal image (**N**). c-Fos-positive cell number in the ARC (**D**), VMN (**J**) and CeA (**O**). Percentage of β-end activated cells in the ARC (**E**) and their correlation with food intake (**F**). Correlation between number of c-Fos cells in the CeA and food intake (**P**). **D**,**J**, ANOVA p<0.05, Tukey's post-test, *p<0.05. **E,** ANOVA, p=0.006, Tukey's post-tests *p<0.05 and **p<0.01. **O**, ANOVA, p=0.0003, Tukey's post-tests, **p<0.01 and ***p<0.001. All data are shown as mean±SEM.

Figure 5. Effects of chronic injections of *E.coli* proteins in mice. A,B. Effects of bi-diurnal injections during 6 days, of *E.coli* proteins from Exp and Stat growth phases or PBS as a control (Contr) in mice on: body weight (**A**), food intake (**B**), meal size (**C**) and meal number (**D**). Total mean food intake was also analyzed separately in light (**E**) and dark (**F**) periods. The satiety ratio, expressed as post-meal interval (s) x1000 vs. food (g) consumed in the preceding meal, at day 1 (**G**) and day 6 (**H**). **A**, **B**, 2-way RM ANOVA, Bonferroni post-tests Contr. vs Stat., ***p<0.001 and **p<0.01. **C**, Student's t-test Contr. vs. Stat. $^{\#}p$<0.05. **E, F**, K-W test p<0.002, Dunn's post-tests, ***p<0.001 and M-W test $^{\#}p$<0.05. **G,** ANOVA p=0.005, Tukey's post-test, **p<0.01. **H**, Student's t-test, $^{\$}p$<0.05. All data are shown as mean±SEM.

Figure 6. Effect of ClpB on electrical activity of ARC POMC neurons. POMC-eGFP neuron (arrow) visualized with x60 objective under infrared (**A**) and fluorescent light (**B**) during patch-clamp recording. Action potential frequency expressed in percent of change vs. basal level, mean ±SEM (**C**). Representative cell-attached recording of a POMC-eGFP neuron activated by ClpB (1 nM), thick black bar above the trace. Washout: 10 min. RM ANOVA p<0.05, Bonferroni post-tests *p<0.05,**p<0.01.

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