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Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors

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Abdominal pain is common in the general population and, in patients with irritable bowel syndrome, is attributed to visceral hypersensitivity. We found that oral administration of specific *Lactobacillus* strains induced the expression of μ-opioid and cannabinoid receptors in intestinal epithelial cells, and mediated analgesic functions in the gut—similar to the effects of morphine. These results suggest that the microbiology of the intestinal tract influences our visceral perception, and suggest new approaches for the treament of abdominal pain and irritable bowel syndrome.

Gut microbiota are composed of different bacterial species, which are involved in the metabolism of nutrients, the maturation of the intestinal epithelium, vasculature and lymphoid tissue, and protection from pathogens[1,2]. The interest in probiotics as a means to restore and maintain health continues to gain momentum. The administration of certain probiotics may prevent abdominal symptoms observed in irritable bowel syndrome—the most common gastrointestinal disease, with unknown etiology and symptoms such as abdominal pain, bloating and altered bowel habits[3–6]. Therefore, we hypothesized that probiotics may induce the expression of receptors on epithelial cells that locally control the transmission of nociceptive information to the intestinal nervous system. Promising candidates include opioid (μ-, δ- and κ-) receptors and cannabinoid receptor 2 (CB2) (refs. 7,8), which have been shown to exert analgesic effects and to have anti-inflammatory functions in several experimental models of colitis[8–11].

We first evaluated the ability of five well known and representative probiotic bacteria belonging to the *Lactobacillus* and *Bifidobacterium* genera (*L. acidophilus* NCFM, *L. salivarius* Ls-33, *L. paracasei* Lpc-37, *B. lactis* Bi-07 and *B. lactis* Bl-04), compared to commensal and adherent-invasive (LF82) *Escherichia coli*, to induce the expression of analgesic receptors. *L. acidophilus* NCFM and *L. salivarius* Ls-33 induced a sustained increase of *OPRM1* mRNA expression in human HT-29 epithelial cells, starting 1 h after bacterial stimulation (**Fig. 1a**). This induction was of the same magnitude as that observed in HT-29 epithelial cells stimulated for 2 h with a positive control (TNF-α; ref. 7). The other probiotics, *L. paracasei* Lpc-37, *B. lactis* Bi-07 and *B. lactis* Bl-04, and the two *E. coli* strains were ineffective (**Fig. 1a**). We found no increase in the expression of δ- and κ-opioid receptors in bacteria-stimulated HT-29 epithelial cells. Concerning cannabinoid receptor expression, only the *L. acidophilus* NCFM strain was able to induce significant *CNR2* mRNA expression compared to that observed in resting epithelial cells ($P < 0.01$, **Fig. 1a**). We observed no induction of *CNR2* mRNA expression in TNF-α–stimulated HT-29 epithelial cells. The inducible effect of NCFM on *OPRM1* and *CNR2* expression in epithelial cells was equally reproduced when we used bacteria killed by 80 °C heat (**Fig. 1b**). Mouse *Rela*$^{-/-}$ embryonic fibroblasts lost their ability to respond to NCFM stimulation, compared to wild-type cells, illustrating the essential role of the NF-κB pathway in the induction of *OPRM1* and *CNR2* by this strain. Next, we conducted a series of *in vivo* experiments to investigate the expression and function of MOR and CB2 in mice and rats, using the live NCFM strain. In an immunohistochemistry analysis using antibodies to opioid receptor μ1 (MOR1) and CB2, we detected the expression of both these receptors in HT-29 epithelial cells incubated with the NCFM strain (**Fig. 1c**). To confirm these results *in vivo*, we orally administered NCFM, at a clinically relevant concentration (10^9 colony-forming units (CFU) per day for 15 consecutive days), to Balb/c mice and Sprague-Dawley rats. We found a rapid and sustained enhancement of NCFM DNA in the feces of mice and rats (**Fig. 1d**). In the colonic section of untreated mice and rats, we detected MOR1 and CB2 expression in approximately 0–20% of epithelial cells; in contrast, the administration of NCFM induced the expression of these proteins in 25–60% of epithelial cells (**Fig. 1e**,**f**). Over the 15 d during which live NCFM was administered, we observed that the changes in weight and the duration of oro-anal transit were similar in all treated and untreated rodents. We found no macroscopic or histological alteration in NCFM-treated rodents, demonstrating that NCFM does not induce adverse effects in the intestinal tract.

To determine the functional role of NCFM-induced analgesic receptors (**Fig. 2a**), we assessed the visceral perception of rats using

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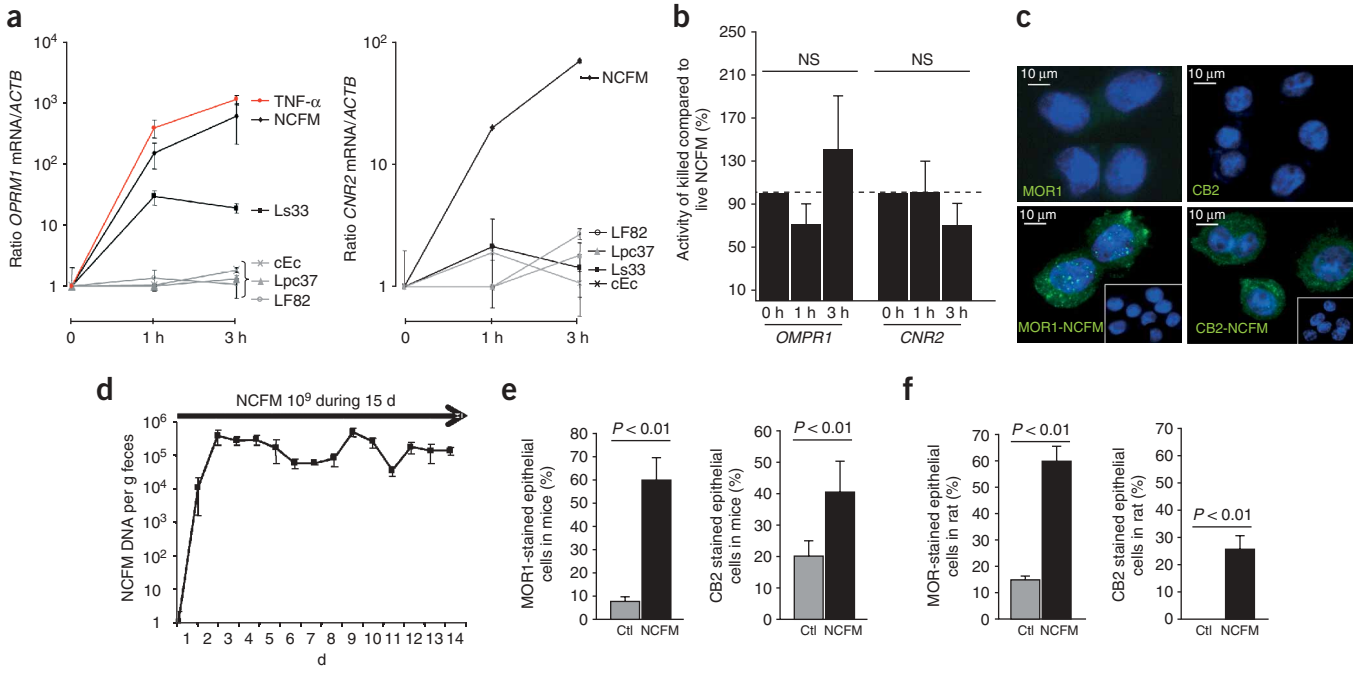

Figure 1 *Lactobacillus acidophilus* NCFM induces the expression of MOR1 and CB2 in epithelial cells. (**a**) Human HT-29 epithelial cells were grown in monolayers, incubated in 5% CO_2 at 37 °C and 95% relative humidity, and stimulated for 1 h or 3 h with different strains of live bacteria (100 CFU/cell) or conditioned with TNF-α (positive control; 2 h, 10 ng/ml). Levels of *OPRM1* and *CNR2* mRNA were quantified by real-time PCR and are normalized to *ACTB* expression. Five experiments, performed in triplicate. NCFM, *L. acidophilus* NCFM; Ls33, *L. salivarius*; Lpc37, *L. paracasei*; cEc, commensal *E. coli*; LF82, adherent-invasive *E. coli*. (**b**) Levels of *OPRM1* and *CNR2* mRNA in human HT-29 epithelial cells stimulated for 1 h or 3 h with 100 CFU/cell heat-killed NCFM (80 °C for 15 min), compared with levels in human HT-29 epithelial cells stimulated under the same conditions by live NCFM (100 CFU/cell). Three experiments, performed in triplicate. NS, not significant. (**c**) Representative MOR1 and CB2 staining in unstimulated HT-29 cells (top) and in HT-29 cells stimulated by live NCFM (3 h, 100 CFU/cell; bottom). Nuclear staining in blue was performed with Hoescht 33342 solution. Insets, negative result of control experiment (an irrelevant antibody was used in NCFM-stimulated HT-29 cells). (**d**) Bacterial DNA was isolated from stool samples of rats and collected over the 15 d of NCFM administration (10^9 CFU/d). NCFM DNA was quantified by real-time PCR. The amount of NCFM in the feces increased rapidly 2 d after oral administration of NCFM and remained stable (at $1.74 \pm 1.6 \times 10^5$ NCFM DNA per g of feces) over the course of administration. (**e**,**f**) Percentage of MOR1- and CB2-stained epithelial cells in the frozen colonic section of (**e**) mice ($n = 8$ per group) and (**f**) rats ($n = 10$ per group) that received NCFM or vehicle (Ctl). All results are expressed as mean ± s.e.m.

a validated technique: colorectal distension[3,4,12]. In untreated rats, a mean colorectal distension of 50 ± 2 mm Hg is required to induce pain, characterized by clearly visible abdominal contractions and elevation of the hindpart of the animal's body[12]. Oral administration of the NCFM strain (10^9 CFU/d) during 15 d decreased normal visceral perception, allowing a 20% increase in the pain threshold ($P < 0.01$; **Fig. 2b**). This analgesic effect was enhanced as the NCFM dosage was increased from 10^7 CFU/d to 10^9 CFU/d ($P = 0.002$; **Fig. 2c**). At a concentration of 10^9 CFU/d, the analgesic effect was induced rapidly within 10 d, maintained for the duration of the treatment (10, 14 or 21 d; **Fig. 2d**) and disappeared 3 d after the last NCFM administration (**Fig. 2e**). In a model of chronic colonic hypersensitivity, elicited by butyrate enemas and mimicking irritable bowel syndrome (**Fig. 2a** and ref. 12), the hypersensitivity of rats was improved by the NCFM strain: treatment with NCFM increased the colorectal distension threshold by 44% compared to that in untreated rats ($P < 0.01$; **Fig. 2f**). In this model, NCFM resulted in an antinociceptive effect of the same magnitude as that caused by the subcutaneous administration of 1 mg morphine per kg (body weight) (**Fig. 2f**). Moreover, it enhanced by 65% the suboptimal analgesic effects of 0.1 mg/kg morphine ($P < 0.001$; **Fig. 2g**). NCFM-induced analgesia was significantly inhibited by peritoneal administration of the CB2-selective antagonist AM-630 (3 mg/kg, Tocris; $P < 0.001$) but not by the opioid receptor antagonist naloxone methiodide (2 mg/kg, Sigma) (**Fig. 2h**), providing indirect evidence for a physiological role of CB2 in the control of intestinal pain.

These results advance our understanding of visceral pain—traditionally viewed as being transmitted by neural mechanisms[13]—by showing that direct contact of NCFM with epithelial cells is able to induce, through the NF-κB pathway, MOR1 and CB2 expression and to contribute to the modulation and restoration of the normal perception of visceral pain. The mechanisms underlying this activity remain unknown. *L. acidophilus* NCFM is a well known probiotic: it is readily isolated from human feces and has been widely investigated for its physiological, biochemical and fermentative properties[14]. Moreover, it possesses unique genomic sequences within the probiotic family[15]. Further investigations will determine their potential involvement in the regulation of MOR1 and CB2 expression. The similar efficacy, in treating pain, of orally administered *L. acidophilus* NCFM and a standard dosage of morphine suggests that specific modulation of intestinal flora may be a promising, safe and relatively inexpensive new treatment for abdominal pain, a prominent symptom of irritable bowel syndrome, which affects 20% of the general population.

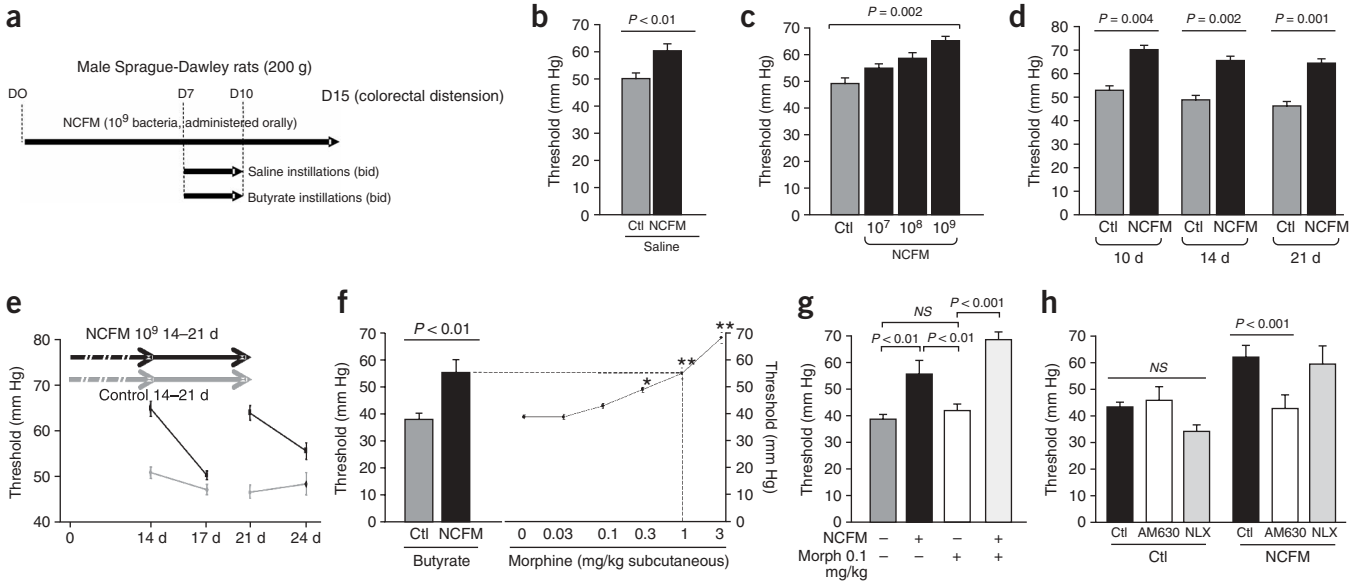

Figure 2 Functional role of *L. acidophilus* NCFM–induced analgesic receptors. (**a**) Design of the functional experiment. We administered live NCFM (10^9 CFU/d over 15 consecutive days) to rats with or without colonic hypersensitivity (butyrate instillations or saline instillations, respectively, between days 7 and 10). (**b**) Pain threshold in 20 rats that were treated with NCFM or received the vehicle (Ctl). (**c**) Pain threshold in 40 rats receiving NCFM (10^7–10^9 CFU/d over 15 days) or a control (Ctl). $n = 10$ rats per group. The pain threshold increased with increasing dosages of NCFM; at a dosage of 10^9 CFU/d, the threshold was significantly different from that in control rats. (**d**) Pain threshold after colorectal distension in 60 rats receiving daily administration of NCFM (10^9 CFU/day for 10, 14 or 21 d) or a control (Ctl). $n = 10$ rats per group. Continuous treatment with NCFM for 10, 14 or 21 d led to a sustained analgesic effect that was similar in all NCFM-treated groups. (**e**) We assessed the duration profile of the NCFM-induced analgesic effect, by repeatedly measuring the pain threshold after 14 or 21 d of NCFM or control. The analgesic effect was transitory, disappearing 3 d after NCFM ceased. $n = 40$. (**f**) Pain threshold in rats ($n = 20$) with colonic hypersensitivity and treated with NCFM (10^9 CFU/d for 15 d) or control (Ctl). Compare these data with pain threshold in rats ($n = 20$) with colonic hypersensitivity that were treated with NCFM or received increasing subcutaneous dosages of morphine. $^*P < 0.05$ and $^{**}P < 0.01$, compared with rats not treated with morphine. (**g**) Pain threshold in control rats and rats with colonic hypersensitivity receiving NCFM (10^9 CFU/d, 15 d), morphine (0.1 mg/kg, subcutaneously, 30 min before colorectal distension) or NCFM + morphine. $n = 10$ rats per group. (**h**) Pain threshold in rats ($n = 10$ per group) with colonic hypersensitivity, receiving NCFM (10^9 CFU/d, 15 d) or control (Ctl), and treated with either a CB2 antagonist (AM630, white), a MOR1 antagonist (naloxone methiodide, NLX, gray) or neither (black). AM630 and NLX were used at optimal dosage and injected peritoneally 30 min before colorectal distension. All results are expressed as mean ± s.e.m.

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AUTHOR CONTRIBUTIONS
C.R. conducted and performed the *in vitro* and *in vivo* experiments. X.T., L.D., C.D. and E.M. contributed to the *in vitro* and *in vivo* experiments. M.C. was involved in the *Rela*$^{-/-}$ experiment. A.G. and D.A. performed colorectal distension in rodents. N.B. and C.N. were involved in the bacteriological experiment. K.G. performed the histological experiments. D.C. was involved in the research proposals and in the coordination of the studies within Danisco research centers. A.C.O. quantified NCFM DNA in the feces using PCR. G.L. validated the taxonomical analysis of the different bacterial species used in this study and performed measurements of *Lactobacillus acidophilus* NCFM products. J.-F.C. contributed to data discussion and analysis. P.D. designed and supervised the study, and wrote the manuscript.

COMPETING INTERESTS STATEMENT
The authors declare that competing financial interests (see the *Nature Medicine* website for details).

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