Immunostimulatory effect of faecal *Bifidobacterium* species of breast-fed and formula-fed infants in a PBMC/CaCO-2 coculture system

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Short title: *Bifidobacterium* spp. and cytokine production *in vitro*

Keywords: *Bifidobacterium* spp.; Caco-2 cells; PBMCs; cytokines; breast feeding; formula feeding; infant's microbiota.

Abstract

Bifidobacterium spp. typical of the human intestinal microbiota are believed to influence the balance of immune responses in the intestinal mucosa.

Aim: To investigate the effect of different bifidobacterial species and mixtures of them in *in vitro* experiments with PBMCs and CaCo-2 cells.

Methods: *Bifidobacterium adolescentis*; *Bifidobacterium angulatum*; *Bifidobacterium breve*; *Bifidobacterium catenulatum*; *Bifidobacterium infantis*; *Bifidobacterium longum*; and two combinations of these bifidobacteria simulating the species composition found in fecal samples from breast fed (BF) and formula fed (FF) infants were used. The levels of several cytokines were measured by direct stimulation of PBMCs and by stimulation of a Caco-2/PBMCs co-culture with bifidobacteria. *Results: B. catenulatum* and *B. breve* were the strongest enhancers of IFN-γ, production by direct stimulation of PBMCs. *B. longum* was the highest inducer of IL-10 and the lowest TNF-α stimulus. In the Caco-2/PBMC system, *B. breve* was the highest inducer of IL-8 production by Caco-2 cells; significantly different from *B. infantis, B. adolescentis* and the FF mixture ($p<0{\cdot}05$). IFN-γ produced by PBMCs stimulated with the BF mixture (containing 22% *B. breve,* compared to 7% in FF mixture) was significantly higher compared to *B. adolescentis*, *B. infantis*, and *B. longum*. *B. adolescentis* also inhibited IFN-γ production compared to FF mixture and *B. longum*. *Conclusions*: The proportion of different *Bifidobacterium* strains seems to be an important determinant of the cytokine balance in the simulated intestinal environment studied. *B. breve* and the combination of the *Bifidobacterium* species typically found in the microbiota of breast-fed infants have shown the most significant effects.

Abbreviations : PBMCs, peripheral blood mononuclear cells; BF, breast fed; FF, formula fed; IECs, intestinal epithelial cells; MRS agar, de Man Rogosa and Sharpe agar; RPMI-1640 medium, Roswell Park Memorial Institute 1640 medium; EMEM médium, Eagle's minimal essential médium; FBS, fetal bovine serum; P/S, penicillin-streptomycin; NAA, non-essential amino acids; TER, Transepithelial Resistance.

Introduction

The intestinal microbiota plays a pivotal role in human health by preventing pathogen colonization, and shaping and maintaining normal mucosal immunity [1]. To preserve this beneficial relationship, the immune system should remain hypo-responsive to commensal bacteria (mucosal tolerance) [2,3], but , at the same time, it has to combat pathogenic bacteria [3]. The breakdown of the delicate balance of the intestinal immune responses causes the development of disease states with bowel inflammation [3]. In this context, intestinal epithelial cells (IECs) play an important role in immune homeostasis [4,5]. IECs are thought to contribute to immunomodulation of mucosal leucocytes by at least two different mechanisms [6], by acting as a physical barrier between gut luminal content (including bacteria) and the underlying immune cells, and by transmitting signals coming from the intestinal content and microbiota to the resident mucosal immune system [4]. IECs secrete many mediators involved in protective responses against potentially pathogenic organisms, such as defensins, mucins, chemokines and cytokines [5].

Bifidobacteria, which are important components of the human intestinal microbiota particularly of breast-fed infants [7], have shown capacity to modulate cytokine production by IECs, monocyte derived dendritic cells and peripheral blood mononuclear cells (PBMCs) in *in vitro* experiments [1,8,9]. In addition, the differences observed in the composition of bifidobacterial species of the intestinal microbiota of breast-fed (BF) and formula-fed (FF) infants have been suggested to influence the incidence of immune-mediated diseases [7,10]. These findings have led to propose the use of some *Bifidobacterium* strains as potential probiotics in the prevention and treatment of pathologies with underlying immune alterations, such as inflammatory bowel diseases, allergy and celiac disease [11-13].

Following all of the aforementioned facts and hypothesis, the objective of this study was to investigate the effect of strains of different bifidobacterial species (*Bifidobacterium adolescentis*; *Bifidobacterium angulatum*; *Bifidobacterium breve*; *Bifidobacterium catenulatum*; *Bifidobacterium infantis*; *Bifidobacterium longum*) and mixtures of them, corresponding to the typical microbiota present in feces from BF and FF children, on the modulation of the cytokine production by IECs and PBMCs in an *in vitro* co-culture system, simulating the intestinal environment .

Materials and methods

Bacteria

The following strains of six different *Bifidobacterium* species were individually evaluated: *B. adolescentis* ATCC 15703, *B. angulatum* ATCC 27535, *B. breve* ATCC 15700, *B. catenulatum* LMG 11043, *B. longum biovar infantis* LMG 11046T and *B. longum biovar longum* ATCC 15707. *B. adolescentis* ATCC 15703, *B. angulatum* ATCC 27535, *B. breve* ATCC 15700, *B. catenulatum* LMG 11043, *B. longum biovar infantis* LMG 11046T and *B. longum biovar longum* ATCC 15707. In addition, two combinations of these bifidobacteria were also used to simulated the percentage of each species in the microbiota from breast-fed (BF) and formula-fed (FF) infants (10). The BF mixture included: *B. infantis* (59·0%), *B. breve* (21·6%), *B. longum* (13·5%), *B. catenulatum* (3·5%), *B. angulatum* (1·8%) and *B. adolescentis* (0·6%); and the FF mixture included: *B. infantis* (62·1%), *B. catenulatum* (14·8%), *B. longum* (10·9%), *B. breve* (7·2%), *B. adolescentis* (5·0%) (no *B. angulatum)*.

Bifidobacteria were grown routinely in MRS agar (Scharlau Chemie SA, Barcelona, Spain) with 0.05% cysteine broth and incubated at 37°C under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK) for 22 h. Cells were harvested by centrifugation (6000 g for 15 min) till stationary growth phase, washed two times in PBS (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4, and resuspended in PBS containing 20% glycerol). Aliquots of these suspensions were frozen in liquid nitrogen and stored at –80°C until used. The number of live cells after storage was determined by CFU counting on MRS-C after 48 h incubation in optimal conditions. For all strains tested, >90% cells were alive upon thawing. One fresh aliquot was thawed for every new experiment to avoid variability in the cultures between experiments.

Leukocyte isolation and bacterial stimulation of PBMCs

Human peripheral blood mononuclear cells (PBMCs) from 7 healthy volunteers were isolated from heparinised blood samples using standard Ficoll gradient centrifugation (Lymphocyte isolation solution, Rafer, Spain). Separated PBMCs were washed twice with RPMI-1640 medium (Bio-Whittaker, Verviers, Belgium) and suspended in the same medium, supplemented with heat-inactivated fetal bovine serum (FBS) (100 ml/l) (BioWhittaker®), after decomplementation, and containing 1% penicillin/streptomycin (P/S) (5000 IU/ml, 5000

mg/ml) (BioWhittaker®). PBMC suspension was adjusted to 2 x 10^6 cells/ml, and 1 x 10^6 cells were used per well in all experiments.

Live bacterial cell suspensions of each individual *Bifidobacterium* strain or the combinations representing the fecal microbiota composition of the BF and FF infants were washed in culture medium and incubated at a final concentration of 10^7 cfu/mL with PBMCs (proportion bacteria:PBMC, 10:1) during 48 hours (5% CO_2, 37ºC). The supernatant was collected, centrifuged and frozen in aliquots at -80ºC until cytokine analysis.

Coculture Caco-2/PBMC and bacterial stimulation

The colonic adenocarcinoma cell line Caco-2 (ECACC Nº 86010202, Salisbury, UK) was cultured at 37ºC and 5% CO_2 in EMEN medium (BioWhittaker®) supplemented with 10% FBS (BioWhittaker®), 1% non-essential amino acid solution (NAA) (BioWhittaker®), 1% L-Glutamine (BioWhittaker®) and 1% P/S (BioWhittaker®). Caco-2 cells were seeded at a density of 8 x 10^4 cells/well in standard 24-well culture plates, and at 4 x 10^4 cells/well on 12 mm inserts in 24-well cell culture plate assemblies (Millipore) with a semipermeable polyethylene terepfthalate membrane (PTE; 1 μm pore size). During cell growth and differentiation, medium was changed every two or three days. The experiments were performed 10-11 days after seeding, once the cells were confluent and differentiated. Confluence was followed with microscopic visualization and TER measurements (Millicell ERS Ohmmeter, Millipore, Madrid, Spain).

Cocultures of the bifidobacteria with Caco-2 cells and PBMCs from healthy donors were performed in 7 different experiments. To that end, a transwell cell culture system was used as described above. Caco-2 monolayers were challenged by apical addition of 2 x 10^6 cfu/insert of a *Bifidobacterium* strain or a combination of strains corresponding to the species composition in fecal samples from BF and FF infants. 500 μL of a PBMCs suspension was added at a concentration of 2 x 10^6 cells/mL in the basal compartment of the culture well for a 12-hour incubation. Thereafter, further 36-hour incubation was allowed after disassembly of the system. In order to measure the cytokine production by the sensitised Caco-2 and PBMCs separately, the basolateral compartment of the Caco-2 cells was replenished with fresh culture medium. After the incubation period, culture media, both from the separated PBMC and Caco-2 cell plates, were collected and frozen in aliquots at -80ºC. PBMCs supernatant was centrifuged prior to freezing to avoid cell presence in aliquots.

Two more conditions, which served as a control of the Caco-2 cell conditioning by the underlying PBMCs, were carried out in two different wells: mixture BF and mixture FF were added to Caco-2 monolayers in transwells with no PBMCs in the basal compartment.

Cytokine quantification in culture supernatants

TNF-α, IL-1β, IL-10, IL-8 and IL-6 cytokines were measured in Caco-2 cells' basolateral medium, and TNF-α, IFN-γ, IL-6, IL-10, IL-2 and IL-4 were measured in the PBMCs supernatant. All cytokines were performed by Cytometric Bead Array System (CBA, BD Biosciencies; Inflammation Kit and either Th1/Th2 kit or Flex set), according to the manufacturer's protocols, and analyzed by flow cytometry (FACScalibur, BD Biosciencies). Data were analyzed using Cellquest software (BD Biosciencies). CBA limit of detection for each cytokine was: IFN-γ: 7·1 pg/ml; TNF-α: 2·8 pg/ml; IL-10: 0·13 pg/ml; IL-6: 1·6 pg/ml; IL-8: 1·2 pg/ml; IL-4: 2·6 pg/ml; IL-2: 2·6 pg/ml; IL-1β: 7·2 pg/ml. IFN-γ was also measured with high sensitivity Immunoassay xMAP Technology (Millipore, Spain) in a Luminex 100 equipment, with a sensitivity of 0·29 pg/ml.

Statistical analyses

Statistical analyses were performed using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). To establish the homogeneity of variances and the distribution of the data, the Levene test was run. As a result of the non-normal distribution of the data and the non-homogeneity of the variances, Mann-Whitney *U* test was used to assess the effect of every experimental condition compared to the other conditions. Data are expressed as medians and quartiles. Significant differences were established at $P < 0{\cdot}05$. Correlations between different bacterial stimulatory conditions were analysed by Spearman's correlation test and considered significant at a P level $< 0{\cdot}05$.

Results

1) Cytokine production by PBMCs cultured with bifidobacteria

In order to determine the immunological effect of bifidobacteria on PBMCs, the production of IFN-γ, TNF-α, IL-10, IL-6, IL-4 and IL-2 was measured in the supernatants of PBMCs cultured in direct contact with the different *Bifidobacterium* strains (individually or mixed). Among all cytokines analysed, only IL-2 was not stimulated (Table 1 and Fig. 1A), with levels below 20 pg/ml (except for the positive control with PHA; data not shown). All the other cytokines were significantly stimulated by all bifidobacterium species and mixtures (compared with the control with only medium).

Regarding IFN-γ production (Table 1 and Fig. 1B), *B. catenulatum* and *B. breve* were the strongest enhancers, followed by FF and BF mixtures (no significant differences were found between both mixtures). *B. catenulatum* induced a higher IFN-γ production than all the other stimuli (except for *B. breve*). *B. breve* induced a higher IFN-γ production than *B. adolescentis*, *B. angulatum* and *B, infantis*, but similar to that induced by *B. catenulatum*, *B. longum* and the mixtures. The total percentage of *B. catenulatum* and *B. breve* was similar in FF and BF mixtures, (22·05% and 25·12%, respectively). This might explain that the levels of IFN-γ produced by PBMCs stimulated with FF and BF mixtures were not statistically different.

B. longum and *B. catenulatum* induced the highest IL-10 production by PBMCs, showing significant differences with IL-10 production in the presence of *B. infantis* and the BF mixture (Table 1 and Fig. 1C). *B. longum* IL-10-induced production was also significantly higher than that of *B. angulatum*, *B. breve* and the FF mixture. The percentages of *B. longum* in FF and BF mixtures were very similar (10·87% vs 13·52%), but *B. catenulatum* was approximately four times higher in FF than BF (14·84% vs 3·50%). The low proportion of *B. catenulatum* and *B. adolescentis*, together with the high proportion of *B. infantis* and *B. breve* in the BF mixture, might explain the significantly lower production of IL-10 induced by the BF mixture compared to that induced by *B. adolescentis*, *B. catenulatum*, and *B. longum* individually (Table 1 and Fig. 1C). Regarding IL-4, *B. catenulatum* also induced a significantly higher production than *B. adolescentis* and *B. infantis* (Table 1 and Fig. 1D).

All *Bifidobacterium* strains stimulated PBMCs to produce very high levels of IL-6, over 4000 pg/ml (Table 1 and Fig. 1E). *B. adolescentis* induced the highest IL-6 production;

significantly higher than *B. angulatum*, *B. breve* and *B. infantis* (P=0·029 in every case). *B. infantis* induced the lowest effect among the assayed strains on cytokine production, not only for IL-6, but also for IFN-γ, IL-10 and IL-4.

With the exception of *B. adolescentis*, all *Bifidobacterium* strains also stimulated PBMCs to produce very high levels of TNF-α (Table 1 and Fig. 1F). A significantly higher TNF-α production was induced by *B. angulatum* and *B. catenulatum* compared to *B. adolescentis*, *B. breve* and *B. longum*. While *B. longum* and *B. adolescentis* induced a high production of IL-10, they both mildly induced TNF-α (Table 1 and Figs. 1C/F). On the other hand, while *B. infantis* and *B. angulatum* induced a mild production of IL-10, they both highly induced TNF-α (Table 1 and Figs. 1C/F).

2) Cytokine production by PBMCs in coculture with Caco-2 cells and bifidobacteria

To analyze cytokine production by PBMCs conditioned by previous co-culture with Caco-2 cells stimulated with bifidobacteria, IFN-γ, TNF-α, IL-10, IL-6, IL-4 and IL-2 were measured in PBMCs supernatants. IL-2 and IL-4 were not detectable and TNF-α was also below the limit or approaching the limit of detection (data not shown). No significant differences in IL-10 and IL-6 production were found, either between different bifidobacteria alone or in mixtures (Table 2 and Figs. 2A/B).

The production of IFN-γ by PBMCs was low in this system (range: 1-93 pg/mL and under the detection limit in 2 of 7 PBMCs donors). Using the available data from the other 5 donors, we found induction of IFN-γ production by BF mixture in 4 of them (>100% vs control) and in 3 of them also with *B. breve* (>50% vs. control), which is singularly high in the BF combination. Moreover, 3 donors showed stimulation with FF (>100% vs. control). BF mixture was the stimulus that induced the highest IFN-γ production (Table 2 and Fig. 2C), significantly higher than *B. adolescentis* (P=0·014), *B. infantis* (P=0·050) and *B. longum* (P=0·047) individually. Although *B. breve* also induced the production of IFN-γ, this effect was not significantly different from the other bifidobacteria (Table 2 and Fig. 2C). *B. adolescentis*' effect on IFN-γ induction was inhibitory relative to the control condition and was significantly different from the stimulatory effect observed with the bifidobacteria mixtures and *B. longum* (Table 2 and Fig. 2C).

3) Cytokine production by Caco-2 cells in coculture with PBMCs and bifidobacteria

To assess the effects of bifidobacteria and bifidobacteria mixtures stimulation on Caco-2 cells in coculture with PBMCs, TNF-α, IL-1β, IL-10, IL-8 and IL-6 cytokines were measured in both apical and basolateral mediums. All cytokines were not detectable in the apical medium, while in the basolateral medium only IL-8 and IL-6 were in a measurable concentration range (IL8: 120-14000 pg/mL; IL6: 30-600 pg/mL). When Caco-2 cells were stimulated with the bifidobacteria alone, with no PBMCs in the underlying compartment the stimulation of both cytokines was 3 to 4 times lower than in the coculture system (data not shown).

When in coculture with PBMCs, *B. breve* highly stimulated the production of IL-6 and IL-8 on Caco-2 cells (66·8% and 45·5%, respectively) (Table 3 and Figs. 3A/B). For IL-8, this production was significantly higher, compared with *B. adolescentis* (P=0·035), *B. infantis* (P=0·025) and FF mixture (P=0·013) (Table 3 and Fig. 3B). Although BF mixture also induced IL-6 and IL-8 production (36·0% and 20·7%, respectively), these values were not significantly higher than those induced by the FF mixture (Table 3 and Fig. 3A/B). No significant differences were observed for IL-6 production between the different stimuli assayed (Table 3 and Fig. 3A).

Considering the PBMCs donors individually, IL-8 and IL-6 production stimulated by the FF mixture was positively and significantly correlated with IL-8 and IL-6 production stimulated by *B. infantis* (P<0·001 for both cytokines). On the other hand, IL-8 production stimulated by BF mixture was correlated with *B. angulatum, B. breve* and *B. catenulatum* (P<0·05), and IL-6 stimulated by BF mixture correlated with *B. adolescentis* and *B. catenulatum* (P<0·05).

Discussion

Bifidobacterium strains have shown capacity to modulate cytokine production by intestinal epithelial cells, monocyte derived dendritic cells and peripheral blood mononuclear cells (PBMC) in *in vitro* experiments [1,8,9]. Trying to define this immunomodulatory capacity seems relevant in order to understand their contribution to the establishment of mucosal tolerance and balanced intestinal immune responses in the early stages of of life. Both these processes have been linked to the prevention of immune-mediated disorders later in life, such as allergies or inflammatory bowel disease [14,15]. Several studies have evaluated the effect of different bifidobacteria in the production of cytokines by Caco-2 cells and PBMCs [6,9,16-18]

but according to our knowledge, this is the first time that the Bifidobacterium strains used in the present work have been employed in coculture experiments and the first time that the mixtures in the proportions of a formula fed and breast fed infant typical microbiota have been used to stimulate these cell types.

In the present study, the levels of several cytokines were measured in two different systems: 1) a direct stimulation of PBMCs with bifidobacteria and 2) a PBMCs/Caco-2 cells co-culture with bifidobacteria stimulating the top layer of Caco-2 cells, which, in turn, can interact with underlying PBMCs through soluble mediators. Reciprocally, PBMCs are able to influence Caco-2 cell activity as well. The profile of cytokine production by PBMC exposed directly to the *Bifidobacterium* strains shows relevant differences compared with the profile of cytokine production by PBMCs in the coculture system, where Caco-2 cells constitute a physical barrier preventing PBMCs' access to the bifidobacteria. The first differential finding is that the level of cytokine production is much lower in the co-culture system. For instance, while 3 out of 6 cytokines measured were above 1000 pg/mL when both bifidobacterial mixtures where used, and 2 out of the remaining 3 gave results higher than 100 pg/mL in direct contact, only IL-6 by PBMCs in the coculture system gave results higher than 1000 pg/mL. It is worth noting that while in the direct contact, IL-6 and TNF-α were the cytokines most highly induced, in the coculture system not only IL-6 but also IL-10 were the cytokines most highly produced by PBMCs. In this sense, Niers et al. (2005) showed in a single culture system that the production of IL-10 by PBMCs is boosted by several *Bifidobacterium* strains and this down-regulates the production of TNF-α and IL-12p70 by these cells. When they used a monoclonal antibody against IL-10, they found a huge increase in the production of these inflammatory cytokines.

Different cytokines (IL-8 and IL-6) were also stimulated on Caco-2 cells, but only when they were previously co-cultured with PBMCs; no cytokine production was measured if the Caco-2 cells were cultured alone with the *Bifidobacterium* strains. Therefore, the presence of PBMCs is an essential factor for the sensitization of Caco-2 cells to respond to bifidobacteria, which is presumably exerted by the communication between both cell types through soluble mediators. In this and other studies Caco-2 cells alone were found to be hyporesponsive to bifidobacteria stimulation [19] and also to other probiotic bacteria [18,19]. Moreover, since cytokine production by Caco-2 cells in the coculture system was only detectable in the basolateral medium and not in the apical one, it demonstrates a polarised secretion by Caco-2 cells, as other authors have found before [18]. In a similar co-culture system, in which Caco-2 cells were stimulated with non-pathogenic *E. coli* and *L. sakei,* an

induction of TNF-α secretion to the subepithelial compartment was observed and this cytokine was signalled as the fundamental candidate for cellular cross talk [18]. In contrast, we found no detectable production of TNF-α, which might be explained by a differential effect from different bacterial species and strains.

Regarding the immunomodulatory effects of specific strains used in these experiments, the most relevant findings have been found regarding the immunostimulatory effects of *B. breve*. This strain stimulated most the production of IL-8 and IL-6 on both Caco-2 and PBMC cells. In the microbiota of breast-fed infants, *B. breve* is the most representative *Bifidobacterium* species (after *B. infantis*, common in all milk fed babies), and this could explain the high IL-8 and IL-6 levels produced by Caco-2 and PBMC stimulated with BF mixture. This link between *B. breve* and BF mixture was supported by the correlation found between IL-8 levels produced by Caco-2 cells stimulated by *B. breve* and BF mixture. Moreover, *B. breve* and BF mixture also stimulated the production of IL-10 and INF-γ by PBMCs (in coculture with Caco-2 cells). All these observations might indicate that the proportion of different *Bifidobacterium* species is an important determinant of the overall contribution to the stimulation of cytokines on the intestinal mucosa. In this sense, it is interesting to note that there was a correlation between the relative inhibition of IL-8 production by Caco-2 cells induced by the FF mixture and by *B. infantis*. It seems that the differences in the proportions of the different strains between the mixtures and the stimulatory/inhibitory capacities shown by the individual strains might explain the results found with their combinations in the BF and FF mixtures.

According to the results, *B. breve* induced a slight pro-inflammatory response, which could turn the mucosal immune system on stand-by and prevent the release of a severe inflammation. It has been already reported that infants from 4 to 6 months old, who daily consumed infant formula fermented with *B. breve* and *Streptococcus thermophilus*, presented less severe episodes of acute diarrhea than the standard formula group [20]. Furthermore, Li and collaborators showed that the administration of *B. breve* to low birth weight infants was useful in promoting the colonization by other bifidobacteria, which might contribute to the establishment of a healthier microbiota [21]. More recently, it has been found that the administration of *B. breve* to preterm infants can up-regulate TGF-β1 signaling and may possibly be beneficial in attenuating inflammatory and allergic reactions in these infants [22].

Regarding the stimulation of the regulatory cytokine IL-10 by PBMCs after direct stimulation with *B.longum*, similar finding have been previously described by Medina et al. (2007), who found that several strains of *B. longum* are strong inducers of IL-10 secretion on PBMCs. On the other hand, the finding that *B. infantis* is a weak inducer of cytokine secretion after direct stimulation of both, PBMCs and Caco-2 cells, is in agreement with prior published results that have described that *B. infantis* attenuates baseline IL-8 secretion in HT-29 epithelial cells [(5)] as well as proinflammatory IL-17 production by murine splenocytes and dextran sodium sulphate-induced intestinal inflammation [(23,24)].

In conclusion, among the *Bifidobacterium* species tested, *B. breve* seems to be the most immunostimulatory strain in a co-culture system resembling the physiological layout of different cell types in the intestinal mucosa. The presence and relative proportions of different *Bifidobacterium* species in the microbiota of breast fed and formula fed infants could be key factors defining the immunomodulatory effect of the gut microbiota in early life.

Acknowledgements

This work has been supported by grants AGL2007-66126-C03-01/ALI and AGL2007-66126-C03-03/ALI, from the Spanish Ministry of Science and Innovation and grants 200570F0091, 200570F0093, and 200870I183 from CSIC. T. Pozo was recipient of a personal grant from the JAE/I3P Program of CSIC (Spain).

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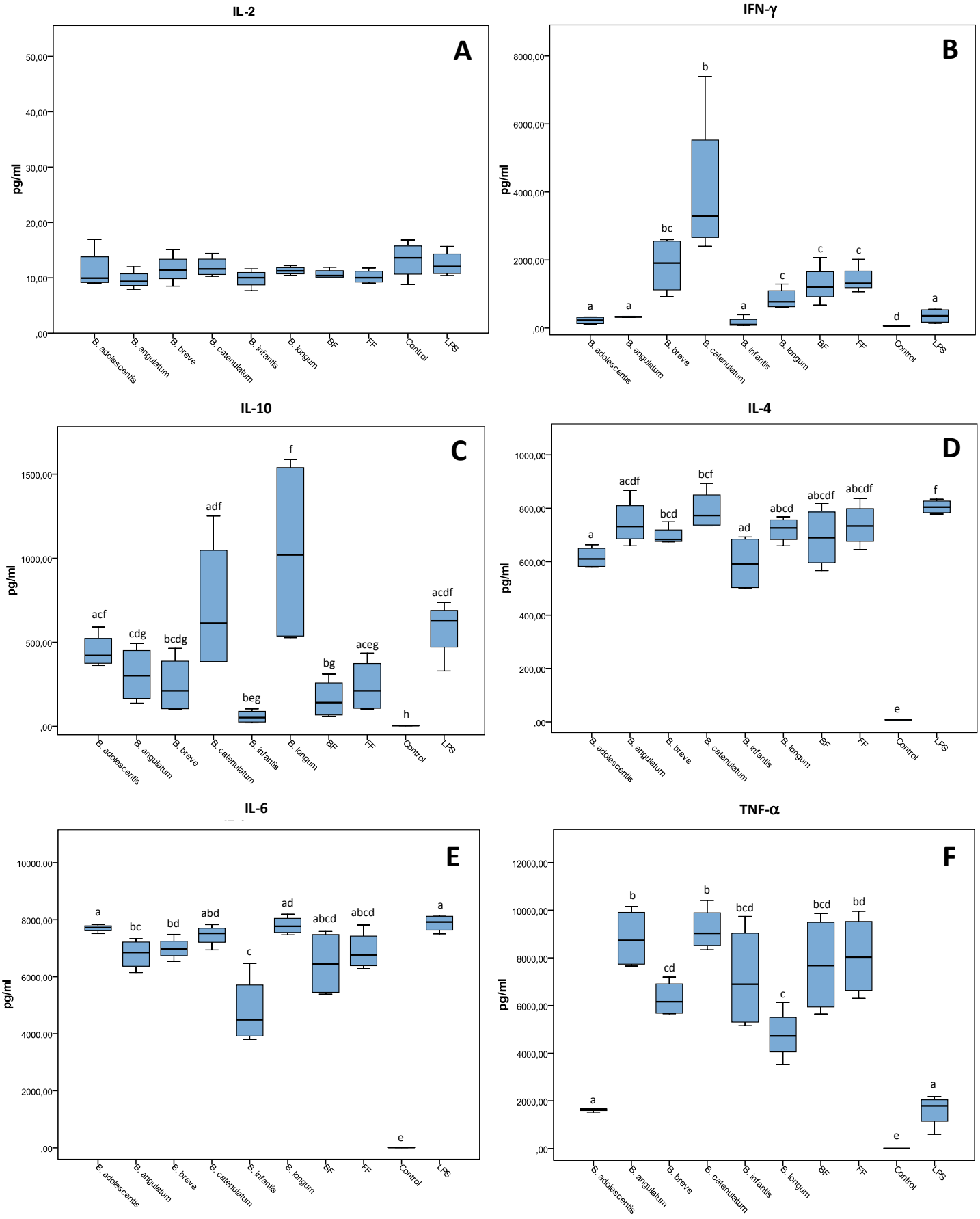

Fig. 1. Cytokine production by PBMCs after 48h incubation with individual bifidobacterium strains and their mixtures (BF and FF) in a 10:1 (bacteria:cell) ratio. Each box represent median (50th percentile) and interquartile range (25th and 75th percentiles). Asterisks and dots represent outliers and extreme values, respectively. Different letters mean statistically significant differences. Mann-Whitney U test. P<0·05. No differences were observed in IL-2 production between conditions.

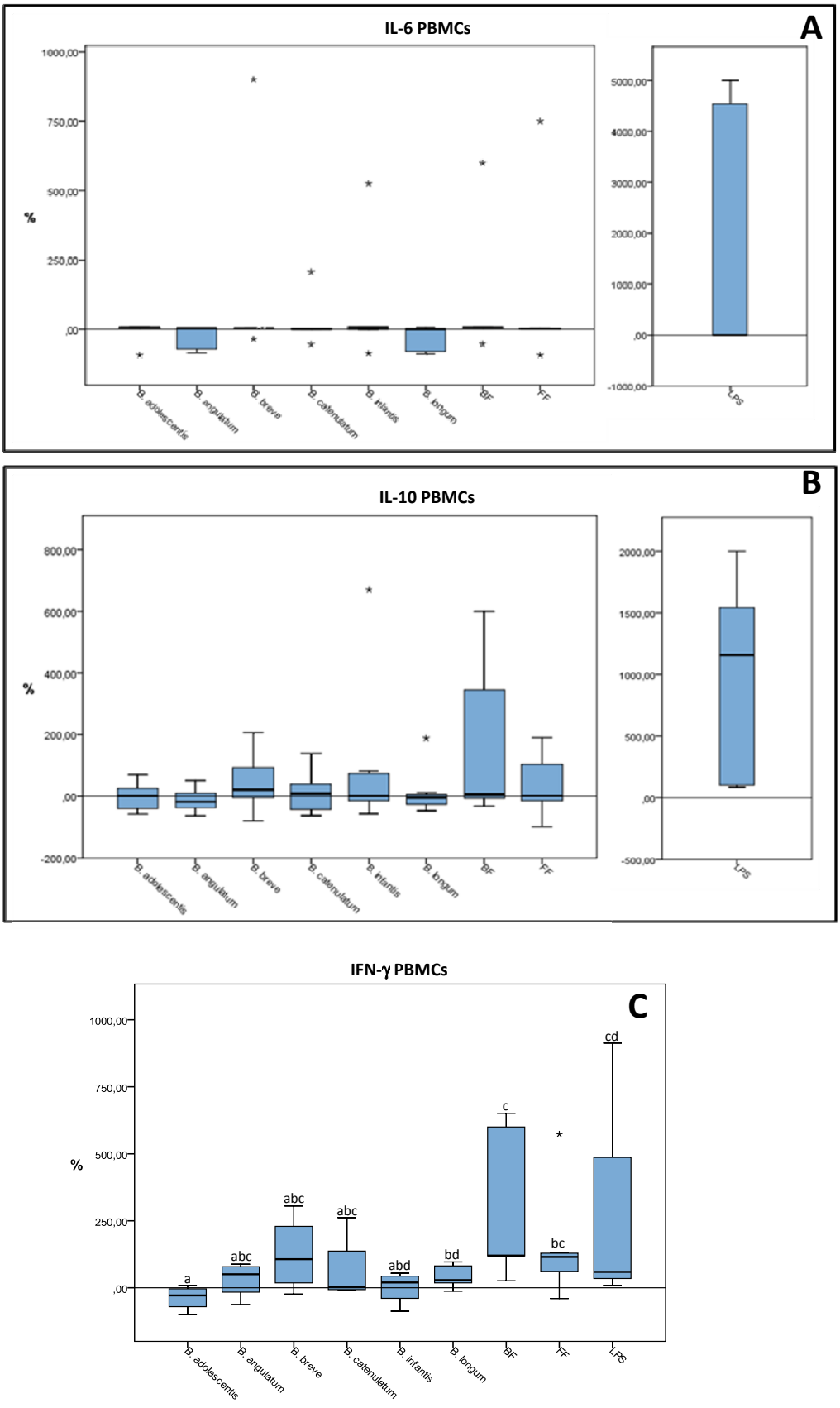

Fig. 2. Cytokine production in a 48-hour culture of PBMCs sensitized by a 12h-incubation in a transwell co-culture system with CaCO-2 cells apically stimulated with bifidobacteria. Values are given as percentage of the control (spontaneous production with no added bacteria). Each box represent median (50th percentile) and interquartile range (25th and 75th percentiles). Asterisks and dots represent outliers and extreme values, respectively. Different letters mean statistically significant differences. Mann-Whitney U test. P<0·05. No differences were observed in IL-10 and IL-6 production between the different *bifidobacterium* conditions employed; however, LPS stimulated production was always significantly higher than the rest of conditions.

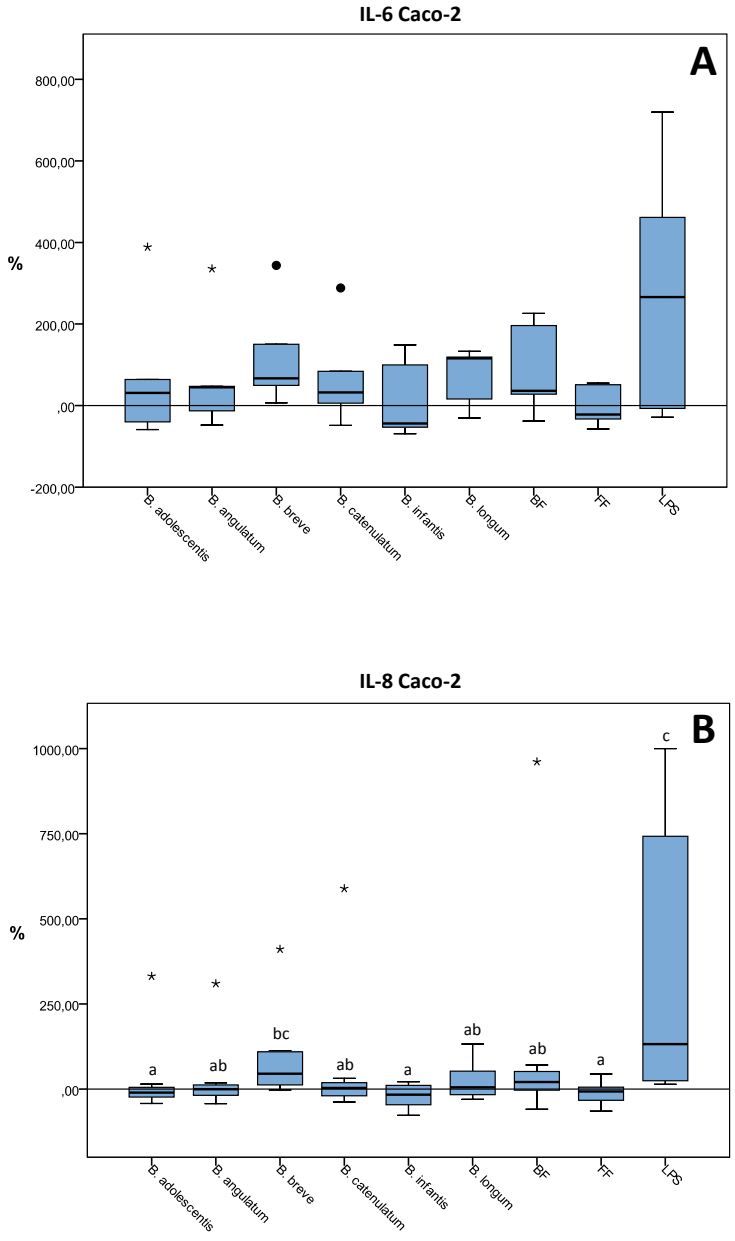

Fig. 3. Bifidobacteria-stimulated cytokine production by Caco-2 cells in a 36-hour culture (basolateral medium) following prior 12h-sensitization with PBMCs in a transwell co-culture system. Values are given as percentage of the control (spontaneous production with no added bacteria). Each box represent median (50th percentile) and interquartile range (25th and 75th percentiles). Asterisks and dots represent outliers and extreme values, respectively. Different letters mean statistically significant differences. Mann-Whitney U test. P<0·05. No differences were observed in IL-6 production between conditions.

Table 1. Cytokine production by PBMCs cultured with the bifidobacteria.

	Cytokine (pg/ml)					
Stimuli	IL-2	IFN-γ	IL-10	IL-4	IL-6	TNF-α
B. adolescentis						
Median	9·95	230·28[a]	422·06[acf]	610·44[a]	7723·24[a]	1656·45[a]
Minimum	9·02	103·34	361·72	579·27	7515·53	1520·79
Maximum	16·94	326·72	591·26	663·11	7840·63	1660·91
B. angulatum						
Median	9·35	329·61[a]	301·70[cdg]	731·17[acdf]	6848·16[bc]	8735·21[b]
Minimum	7·96	316·58	138·39	659·16	6145·95	7652·71
Maximum	11·98	348·84	492·82	867·62	7332·61	10162·08
B. breve						
Median	11·40	1912·91[bc]	211·40[bcdg]	682·70[bcd]	6973·64[bd]	6160·78[cd]
Minimum	8·48	922·97	99·19	673·93	6536·60	5653·84
Maximum	15·13	2598·70	464·38	748·79	7488·86	7193·55
B. catenulatum						
Median	11·60	3292·41[b]	614·68[adf]	772·46[bcf]	7525·74[abd]	9030·84[b]
Minimum	10·30	2401·38	384·15	733·33	6939·44	8338·47
Maximum	14·41	7392·18	1251·51	893·34	7827·05	10418·59
B. infantis						
Median	10·02	100·43[a]	52·38[beg]	591·48[ad]	4490·01[c]	6891·72[bcd]
Minimum	7·65	80·38	20·17	498·56	3801·96	5151·38
Maximum	11·60	390·78	104·47	692·46	6474·54	9737·87
B. longum						
Median	11·26	774·78[c]	1019·33[f]	725·83[abcd]	7770·10[ad]	4722·53[c]
Minimum	10·41	608·80	528·01	659·14	7470·63	3520·22
Maximum	12·20	1291·65	1587·53	767·42	8191·40	6135·92
BF combination						
Median	10·40	1204·56[c]	141·26[bg]	689·54[abcdf]	6446·27[abcd]	7677·01[bcd]
Minimum	10·07	675·42	57·70	566·37	5387·99	5640·80
Maximum	11·93	2073·37	311·39	817·92	7590·74	9865·33
FF combination						
Median	10·02	1316·86[c]	211·71[aceg]	733·19[abcdf]	6766·16[abcd]	8032·24[bd]
Minimum	9·02	1068·30	103·45	644·90	6286·95	6296·63
Maximum	11·78	2020·49	435·16	836·95	7815·05	9958·65
Control						
Median	13·60	60·40[d]	4·87[h]	8·57[e]	10·51[e]	3·27[e]
Minimum	8·79	55·79	3·71	6·14	7·49	1·88
Maximum	16·82	67·65	6·38	10·90	19·11	4·15
LPS						
Median	12·06	360·71[a]	627·62[acdf]	804·01[f]	7920·60[a]	1789·52[a]
Minimum	10·37	141·79	329·47	777·99	7509·36	599·38
Maximum	15·66	553·15	737·32	833·66	8158·12	2183·25

Different superscript letters mean statistically significant differences· Mann-Whitney U test. P<0·05.
BF: breast fed; FF: formula fed.

Table 2. Cytokine production by PBMCs in co-culture with CaCO-2 cells and bifidobacteria.

	Cytokine (%)*		
Stimuli	IL-6	IL-10	IFN-γ
B. adolescentis			
Median	6·36	1·56	-28·76[a]
Minimum	-93·00	-57·20	-100·00
Maximum	9·29	69·74	8·33
B. angulatum			
Median	4·92	-17·77	50·13[abc]
Minimum	-85·18	-62·76	-63·47
Maximum	6·90	52·10	87·66
B. breve			
Median	3·40	21·40	106·50[abc]
Minimum	-35·26	-80·06	-23·63
Maximum	900·00	207·28	305·20
B. catenulatum			
Median	0·85	9·79	4·11[abc]
Minimum	-53·98	-62·13	-10·33
Maximum	207·01	139·51	261·91
B. infantis			
Median	6·30	1·65	19·91[abd]
Minimum	-86·75	-56·60	-87·30
Maximum	525·32	670·52	54·51
B. longum			
Median	-0·33	-4·93	28·57[bd]
Minimum	-88·96	-47·41	-12·57
Maximum	7·11	188·64	96·19
BF combination			
Median	5·92	7·31	120·14[c]
Minimum	-52·77	-30·89	25·78
Maximum	600·00	600·00	651·50
FF combination			
Median	1·52	2·55	114·93[bc]
Minimum	-92·94	-98·83	-40·12
Maximum	750·00	190·03	572·79
LPS			
Median	2·55	1161·25	59·28[cd]
Minimum	-0·12	85·80	9·57
Maximum	5000·00	2000·00	913·30

*Cytokine production presented as percentage of the control (spontaneous production in the co-culture without bifidobacteria).

Different superscript letters mean statistically significant differences. Mann-Whitney U test. $P<0·05$. BF: breast fed; FF: formula fed.

Table 3. Cytokine production by CaCO-2 cells in co-culture with PBMCs and bifidobacteria.

	Cytokine (%)*	
Stimuli	IL-6	IL-8
B. adolescentis		
Median	30·95	-9·97[a]
Minimum	-58·67	-42·07
Maximum	388·74	331·73
B. angulatum		
Median	44·87	-0·35[ab]
Minimum	-47·91	-42·42
Maximum	335·53	310·10
B. breve		
Median	66·83	45·52[bc]
Minimum	6·80	-2·02
Maximum	343·81	411·06
B. catenulatum		
Median	32·36	3·38[ab]
Minimum	-48·50	-37·90
Maximum	288·32	588·94
B. infantis		
Median	-43·60	-16·35[a]
Minimum	-68·78	-76·92
Maximum	148·92	21·75
B.longum		
Median	115·96	5·22[ab]
Minimum	-30·66	-29·73
Maximum	132·98	132·21
BF combination		
Median	36·01	20·67[ab]
Minimum	-38·09	-59·27
Maximum	226·18	961·54
FF combination		
Median	-21·75	-6·81[a]
Minimum	-57·14	-64·90
Maximum	55·13	44·58
LPS		
Median	266·20	132·24[c]
Minimum	-28·51	14·26
Maximum	719·61	1000·00

* Cytokine production presented as percentage of the control (spontaneous production in the co-culture without bifidobacteria).

Different superscript letters mean statistically significant differences. Mann-Whitney U test. $P<0·05$. BF: breast fed; FF: formula fed.