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**THE EFFECTS OF A PROBIOTIC MIXTURE ON NSAID
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10 **THE EFFECTS OF A PROBIOTIC MIXTURE ON NSAID ENTEROPATHY: A**
11 **RANDOMIZED, DOUBLE-BLIND, CROSS-OVER, PLACEBO-CONTROLLED**
12 **STUDY.**
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ABSTRACT

Background. Non-steroidal anti-inflammatory drugs (NSAIDs) can cause serious side gastrointestinal effects. Fecal calprotectin assay represents a simple and practical method for diagnosis of NSAID enteropathy. Intestinal microorganisms are necessary for the development of NSAID-induced small bowel lesions, so it has been suggested that probiotics could protect against NSAID enteropathy.

Aim. To evaluate the effect of a probiotic mixture in respect to placebo on fecal calprotectin concentrations (FCCs) in healthy volunteers receiving indomethacin.

Methods. In a double-blind, cross-over trial, 20 healthy volunteers ingested a daily dose of probiotic mixture (*VSL#3*) or placebo for 21 days. From day 16 to 19 all subjects were also administered 50 mg/day of indomethacin. FCCs were measured the day before starting probiotic/placebo ingestion (T0), and every day within the day 15 and 21.

Results. During dosing with probiotic, median FCCs were significantly increased only at day 17 in respect to T0 values, while during dosing with placebo they were significantly increased at every day from day 17 to day 21 in respect to T0 values.

Conclusions. Treatment with *VSL#3* before and during indomethacin therapy significantly reduces FCCs in healthy subjects in respect to placebo, suggesting that this approach could be useful in decreasing indomethacin-induced intestinal inflammation.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are largely used in clinical settings for their anti-inflammatory, analgesic and antipyretic effects. Nevertheless, they can also cause serious side effects, mainly in the gastrointestinal tract (1). Till now, stomach and duodenum injuries induced by NSAIDs have been well established and described (2). However, there is a growing interest in NSAID side effects on small bowel (the so-called “NSAID enteropathy”), since new endoscopical techniques are now available for the detection of small intestinal lesions (2-3).

It has been showed that the pathogenesis of NSAID enteropathy is multifactorial, involving a combination of biochemical events, represented by inhibition of cyclooxygenase (COX) 1 and 2 and by topical effects of NSAIDs on enterocytes, all responsible for an alteration of mucosal integrity and the disruption of intercellular junctions (2). This results in an increased intestinal permeability, allowing mucosal exposure to a variety of luminal aggressors (bacteria, bile acids, etc.), with consequent inflammatory reactions and macroscopic alterations (3-4).

Different techniques have been used to detect NSAID enteropathy: the recently introduced enteroscopy and capsule endoscopy, and other indirect methods, such as the assessment of intestinal inflammation by the ¹¹¹Indium labeled leucocyte technique or fecal calprotectin measurement, as well as the intestinal permeability tests (3). In particular, Tibble *et al.* showed that the assay of fecal calprotectin concentrations (FCCs) represents a simple, practical and reliable method for the diagnosis of NSAID enteropathy in humans (5).

It has been suggested that intestinal microorganisms are necessary for the development of NSAID-induced small bowel lesions, since “germ-free” animals were found to be resistant to indomethacin injuries (3,6). It has also been showed that NSAID ingestion may disrupt the homeostasis of intestinal flora and may induce the overgrowth of Gram-negative and

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3 anaerobic bacterial species, able to exacerbate the intestinal injury caused by NSAIDs (7-
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8 Probiotics have been defined as “living microbial supplements which beneficially affects the
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10 host animal by improving its microbial balance” (11). It has been showed that the use of
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12 probiotic bacteria may have therapeutic effects in gastrointestinal disorders such as
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14 inflammatory bowel diseases (IBDs) (12-15), irritable bowel syndrome (16), intestinal
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16 infections and antibiotic-induced diarrhea (17-18).

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19 In consideration of these evidences, it has been suggested that modulating the intestinal flora
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21 by probiotics could protect against NSAID-induced enteropathy. Some studies using
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23 different strains of probiotics have been carried out on this topic in animals, but results were
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25 not concordant (19-20). The only study performed in humans, by Gotteland *et al.*, showed
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27 that the regular ingestion of *Lactobacillus* GG (LGG) may protect the integrity of the gastric
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29 mucosal barrier against indomethacin, but has no effect on the intestinal permeability
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31 alterations induced by the NSAIDs (4).

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36 Moreover, uncertainties still remain regarding the probiotic strain to be chosen, the correct
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38 dosage, as well as the possibility of a different efficacy of a probiotic mixture in respect to a
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40 single strain. Just regarding this latter topic, all the above cited works evaluated the probable
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42 protective effect of only a single strain of probiotic. Conversely, different studies showed a
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44 protective role of a combination of probiotic bacteria in gastrointestinal disorders, such as
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46 experimental colitis and Crohn’s disease, whereas a single species of probiotic resulted to be
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48 lower or not effective at all (12,21-23). Therefore, the possibility of a synergistic action
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50 among the individual strains present in a probiotic mixture has been suggested, leading to a
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52 more efficacious protective role on gastrointestinal mucosa (21,24).

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57 The aim of this study is to evaluate the effect of a probiotic mixture in respect to placebo on
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60 FCCs in healthy volunteers receiving indomethacin.

METHODS

Patients and study design

Between June 2008 and June 2009, healthy volunteers were invited to enter the study.

Subjects with IBDs or family history of IBDs, colorectal cancer, coexisting and severe cardiopulmonary, hepatic, renal, neurologic, psychiatric, endocrine and rheumatologic diseases, malignancy, pregnancy, alcohol abuse, other intestinal disorders characterized by increased mucosal permeability and inflammatory changes, age <18 and >75 years, were excluded from the study. All subjects did not take any chronic medications.

In a double-blind, cross-over trial, all the enrolled subjects were asked to ingest randomly a daily dose of a probiotic mixture (VSL#3) or placebo for 21 days. For four days (from day 16 to day 19), all subjects were also administered 50 mg/day of indomethacin. Each subject was asked to provide a stool sample for measurement of calprotectin levels the day before starting probiotic/placebo ingestion (T0), and every day within day 15 and day 21. According to the cross-over design of the study, after an interval period of at least 30 days, all volunteers were given probiotic or placebo, on the basis of the regimen they had previously performed (figure 1).

Compliance was assessed by counting the dispensed and returned sachets, as well as by questioning patients. Compliance rate was calculated by dividing the number of taken sachets by that of the dispensed ones.

Procedures were in accordance with the Ethical standards of the Helsinki Declaration of 1964, as modified by the 48th World Medical Association in 1996. Each subject gave written informed consent to the study. The study was approved by Ethical Committee of the Faculty of Medicine, Catholic University of Rome.

Study medication

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3 Each VLS#3 (VSL Pharmaceuticals Inc., Italy) sachet (4.4 g) contains 900 billion viable
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5 lyophilized bacteria consisting of four strains of Lactobacilli (*L. casei*, *L. Plantarum*, *L.*
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7 *acidophilus*, *L. bulgaricus*), three strains of Bifidobacteria (*B. longuum*, *B. breve* and *B.*
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9 *infantis*), and one strain of *Streptococcus salivaris* subspecies *thermophilus*.

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12 Placebo consisted of corn starch and it was dispensed in bags identical to those containing
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14 probiotic. The taste and the smell of both probiotic and placebo were not readily
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16 identifiable.
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19 20 21 22 ***Fecal Calprotectin Test***

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24 Each subject was instructed to collect and return a single stool sample using a disposable
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26 plastic bucket-type device to avoid toilet water artifacts and simplify laboratory sampling.
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28 Fecal specimens were retrieved within 48h of defecation. Upon receipt, stools were either
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30 aliquoted and immediately assayed or stored at -20°C for subsequent biomarker
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32 determinations.
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36 Stool samples were prepared and analyzed according to the manufacturer's instructions
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38 (Calprest; Eurospital SpA, Trieste, Italy). The weight of each sample (40–120 mg) was
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40 measured and an extraction buffer containing citrate and urea was added in a weight per
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42 volume ratio of 1:50. Samples were mixed for 30 seconds with a vortex method and
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44 homogenised for 25 minutes. One milliliter of homogenate was transferred to a tube and
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46 centrifuged for 20 minutes. The supernatant was collected and frozen at -20°C. In most
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48 cases, time from sampling to preparation and freezing was estimated to be 1 to 3 days.
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50 Before testing, the supernatants were thawed and then analyzed with Calprest, a quantitative
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52 calprotectin ELISA, for calprotectin determination in stools. Calprotectin was expressed as
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54 micrograms per gram of feces.
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3 *Safety Assessments*
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5 A safety assessment was performed on documentation of any adverse events that occurred
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7 during the study period.
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12 *Statistical analysis*
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14 Comparison of FCCs at T0 and FCCs at days 15, 16, 17, 18, 19, 20 and 21, was performed
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16 by means of Wilcoxon Matched Pairs test during dosing with both regimens. Calprotectin
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18 levels were expressed as median value and quartiles (25th, 75th percentiles). A P value of
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20 0.05 or less was regarded as significant.
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24 In consideration of the median and the standard deviation values at T0 and after
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26 indomethacin ingestion, with the population study consisting of 20 subjects, with an α error
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28 of 0.05, the estimated test power is 93%.
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RESULTS

Twenty healthy subjects (M/F:11/9, mean age 35±10 years) entered the study.

Median compliance rate was 100% during dosing with probiotic and 99% during dosing with placebo. At T0, days 15, 17, 19 and 20, all subjects provided their stool samples during dosing with both regimens. During dosing with probiotic, at days 16 and 18, one subject did not provide its stool sample; during dosing with placebo, at day 21, two subjects did not collect their stools.

There were not significant differences between FCCs at T0 and day 15 (before starting indomethacin therapy and after the pre-treatment period with probiotic/placebo) during dosing with both regimens.

Median FCCs at T0 and from day 15 to day 21, were reported in table 1 and figure 2.

During dosing with probiotic, a significant increase of median FCCs in respect to basal value was found only at day 17 ($P<0.05$). Median FCCs relative to day 16, 18, 19, 20, 21 did not significantly differ with respect to FCCs at T0 ($P=$ n.s. for all the comparisons) (table 1).

During dosing with placebo there was a significant increase of median FCCs relative to every day from day 17 to day 21, with respect to FCCs at T0 ($P<0.05$ for all the comparisons) (table 1). Median FCCs relative to day 16 did not significantly differ with respect to T0 values ($P=n.s.$).

Figure 3 shows FCCs at T0 and at day 19 (the last day of indomethacin ingestion) for each individual both during dosing with probiotic and during dosing with placebo.

No adverse events were reported during dosing with both regimens.

DISCUSSION

This study shows that the regular *VSL#3* ingestion before and during the administration of indomethacin in human healthy volunteers, can significantly decrease FCCs in respect to placebo. Our finding suggests that this approach could be useful in decreasing indomethacin-induced intestinal inflammation.

Previous attempts to evaluate the effect of probiotics on NSAID enteropathy has been performed in animals, not reporting concordant results (19-20). An our *in vitro* study showed that a preparation of *Lactobacillus acidophilus* strain LB protected tight junctions of HT-29 cells from aspirin (ASA) damage, suggesting that probiotics could play a role in the prevention of ASA-induced alterations of the intestinal structure (25). The only human study was performed by Gotteland *et al* (4). The Authors enrolled 16 healthy volunteers, performing four tests in each volunteer to evaluate gastric and intestinal permeability: the first test was performed to assess baseline parameters, the second one after an administration of 75 mg of indomethacin the previous night and 50 mg of indomethacin before the test; in the other two tests the same doses of indomethacin were ingested after a daily administration of live or heat-killed LGG, each day for five days. They concluded that the regular ingestion of live LGG could have a protective effect on the integrity of the gastric mucosal barrier against indomethacin-induced injury, but had no effect at intestinal level (4). In this study all subjects were given a single strain of probiotic and for only 5 days; furthermore, it is likely that the daily dose of LGG ingested (2.4×10^9) was not enough to prevent indomethacin-intestinal injury. In addition, as the Authors supposed, the low protein content of the product could result in a decreased buffer capacity for gastric acid, with a consequent reduction of bacteria surviving in the stomach and able to enter in bowel. Finally, it was suggested that the substrates for bacterial growth found in the tested product

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3 were present in such quantities unable to warrant their survival along the whole bowel, thus
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5 decreasing their potential protective effect in the more distal portions (4).
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8 We designed our study to possibly overcome the limits underlined by Gotteland *et al* in their
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10 pioneering work. In particular, as regards doses and time of administration, till now, there is
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12 not a clear knowledge about the relationship between the amount of probiotic bacteria and
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14 the beneficial effects. However, we used an high dosage probiotic preparation, containing
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16 significantly greater concentration of live bacteria (900 billion viable lyophilized bacteria)
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18 than traditional probiotic preparations. About the resistance to gastric acidity, all strains in
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20 our probiotic preparation have been reported to be highly bile and acid resistant, and to reach
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22 surviving the human colon (26). Moreover, it has been speculated that the beneficial effects
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24 of various probiotics may reflect species-specific properties (27) with mechanisms of action
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26 including competition for nutrients and adhesion sites, effects on mucin, immunoglobulin,
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28 and antimicrobial peptide secretion (28,29). Therefore, the advantage to use a probiotic
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30 mixture containing different bacterial strains may derive from the synergistic effects of all
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32 these bacterial species (21). At this regards, an *in vitro* study showed that VSL#3 might be
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34 more effective than the application of a single strain on stabilization of the cytoskeleton and
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36 on induction of mucin expression after a pathogen (*Salmonella dublin*)-induced alterations
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38 (24).
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45 Our study is the first one performed in humans on NSAID enteropathy by using a multi-
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47 strain probiotic formulation, which has been already reported as effective in preventing
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49 relapse of pouchitis (30), development of pouchitis after ileo-anal pouch formation (31), as
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51 well as in maintaining and inducing remission of active ulcerative colitis (15,31).
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54 Finally, in our study design, we included a period of probiotic pretreatment. This choice was
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56 also supported by an animal study by Watanabe *et al* (19). The Authors showed that, in rats,
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58 a single dose of viable probiotic was not able to inhibit intestinal injury, whereas a one-week
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3 pre-treatment prevented indomethacin-induced intestinal damage. This finding may suggest
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5 that a period of probiotic pre-treatment is necessary to prevent intestinal injury. In our study
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7 we administered our probiotic formulation for 21 days, and in particular for 15 days before
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9 the indomethacin ingestion. It is possible that the probiotic pre-treatment period we used
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11 could have played a role in achieving our findings. Further studies need to be performed to
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13 clarify whether a pre-treatment period is either necessary or may be reduced.
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17 We showed significantly increased FCCs from the 17th to the 21st day when subjects were
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19 treated with placebo, but also at day 17 when the same subjects were on probiotic treatment.
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21 These findings could indicate that our therapeutic strategy is useful in reducing although not
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23 in totally preventing, indomethacin-induced small bowel inflammation.
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29 In conclusion, for the first time, we showed that a probiotic treatment before and during
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31 indomethacin therapy significantly reduces FCCs in healthy subjects, thus suggesting that
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33 this approach could be useful in decreasing indomethacin-induced intestinal inflammation. It
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35 should be considered that we studied an indirect marker of NSAID enteropathy; therefore,
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37 further and larger studies need to be performed to confirm our results by direct diagnostic
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39 techniques. Moreover, the possible protective role of probiotics should also be investigated
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41 when NSAIDs are used in larger amounts or for a longer period of time.
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6 *VSL#3* and placebo preparations.
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For Peer Review

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Table 1. Median fecal calprotectin concentrations (quartiles) during dosing with both regimens.

	<i>indomethacin plus VSL#3</i>	<i>indomethacin plus placebo</i>
<i>T0</i>	13 (3-25)	6 (2-15)
<i>day 15</i>	6 (2-21)	5 (3-17)
<i>day 16</i>	11 (6-35)	5 (3-17)
<i>day 17</i>	27 (4-42)*	20 (3-25)*
<i>day 18</i>	23 (3-93)	30 (15-90)*
<i>day 19</i>	13 (4-45)	47 (18-99)**
<i>day 20</i>	21 (4-100)	38 (11-86)**
<i>day 21</i>	15 (6-36)	43 (5-81)*

*P<0.05 vs T0; **P<0.01 vs T0 (Wilcoxon Matched Pairs test)

FIGURE LEGENDS

Figure 1. Study design.

Figure 2. Median fecal calprotectin concentrations during the study period.

n values of samples tested for each one of the days: n=20 both in the two tests at T0, days 15, 17, 19 and 20; n=19 during dosing with probiotic and n=20 during dosing with placebo at days 16 and 18; n=18 during dosing with placebo and n=20 during dosing with probiotic at day 21.

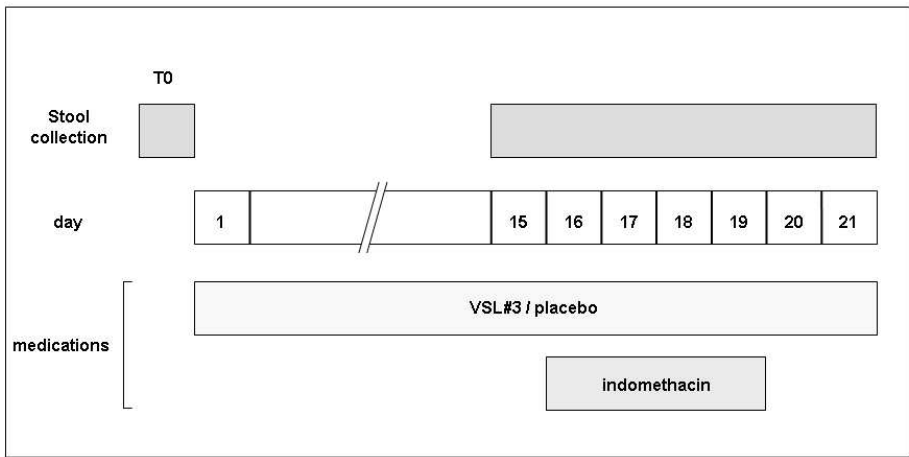
Figure 3. Fecal calprotectin concentrations for each individual at T0 and at day 19 during dosing with both regimens.

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Declaration of personal interest: none. Declaration of funding interests: none.

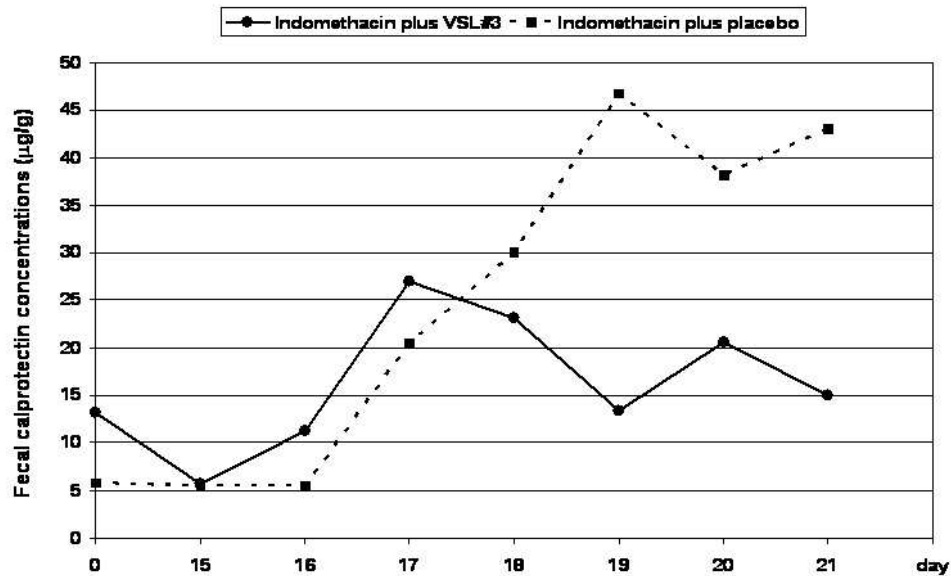
Pre-Review

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Study design.
254x190mm (96 x 96 DPI)

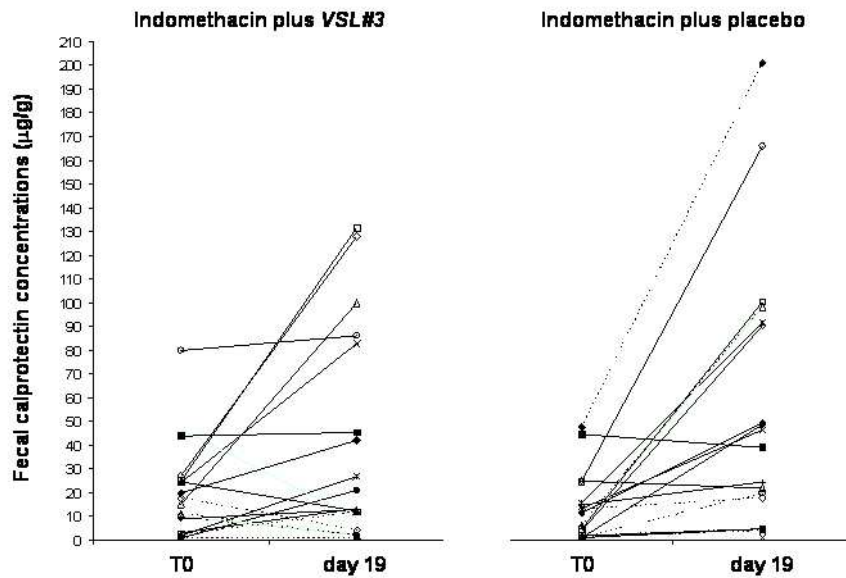
Review



Median fecal calprotectin concentrations during the study period.
n values of samples tested for each one of the days: n=20 both in the two tests at T0, days 15, 17, 19 and 20; n=19 during dosing with probiotic and n=20 during dosing with placebo at days 16 and 18; n=18 during dosing with placebo and n=20 during dosing with probiotic at day 21.

275x190mm (72 x 72 DPI)

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Fecal calprotectin concentrations for each individual at T0 and at day 19 during dosing with both regimens.
275x190mm (72 x 72 DPI)

Review