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Should probiotics be tested on ex vivo organ culture models?

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The use of probiotic strains as nutritional supplements has been gaining ground in the last decade. As the mechanisms with which they modulate innate and adaptive immunity start to unravel, probiotics have repeatedly been suggested as potential treatment for a wide variety of diseases, including inflammatory bowel disease (IBD). However, even though the benefits of probiotic treatment for conditions like atopic dermatitis are well established, very limited clinical benefit has been obtained on IBD treatment. This could be due to the lack of suitable models on which to obtain valid pre-clinical data to select the most appropriate strain for a given condition.

We recently described a newly developed model for the culture and apical stimulation of whole human intestinal mucosal explants. We showed that the tissue was only viable if incubated in an O_2 chamber, but it was possible to stimulate the tissue with bacteria in a conventional incubator. We used the new set-up to test three different Lactobacilli strains, none of which appeared to be benign on inflamed IBD mucosa.

Keywords: IBD, probiotics, postbiotics, ex vivo organ culture

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Introduction

Prebiotics, namely components that facilitate the growth of beneficial microbes, as well as probiotics, have been introduced to the wide public not many years ago, however their popularity increases year by year, and the potential of using them as therapeutic agents for a variety of diseases is being extensively discussed. Promising results have been obtained in various studies using probiotics as adjuvants during broad-spectrum antibiotic treatments or as prophylaxis against post-operative infections[1] and the feedback from the consumers on their use in steady-state couldn't be more enthusiastic. However, when probiotics have been used as the actual treatment agents for induction of remission in IBD, or even to ameliorate IBS symptoms, the outcome has not always been as encouraging.[2,3] Of note, when probiotics were used to treat patients with acute pancreatitis, they significantly increased the mortality rate as opposed to the placebo treatment.[4]

Thus, previous disappointing clinical trials on the IBD front as well as other inflammatory diseases, along with the fact that the molecular mechanisms of interaction between probiotics or their metabolites and the host still remain for the greater part unknown, have led to a reduction in the number of clinical trials performed in the past 3–5 years. Pre-clinical data obtained on the models used so far are not considered sufficient and many concerns have been expressed over the past years, with scientists reporting that the models used to evaluate the effects of various treatments on the intestinal mucosa are not accurate enough, whether they are in vitro models using cell lines or mouse models of colitis.[5,6] True enough, there is great variation to be observed between different protocols employed in different laboratories and the resulting data.[7] Thus, the need for the development of more realistic models enabling an objective assessment of the ensued immune response after application of the treatment has been widely recognized.

The interaction of ingested bacteria such as probiotics with intestinal mucosa is complex and comprises several key events

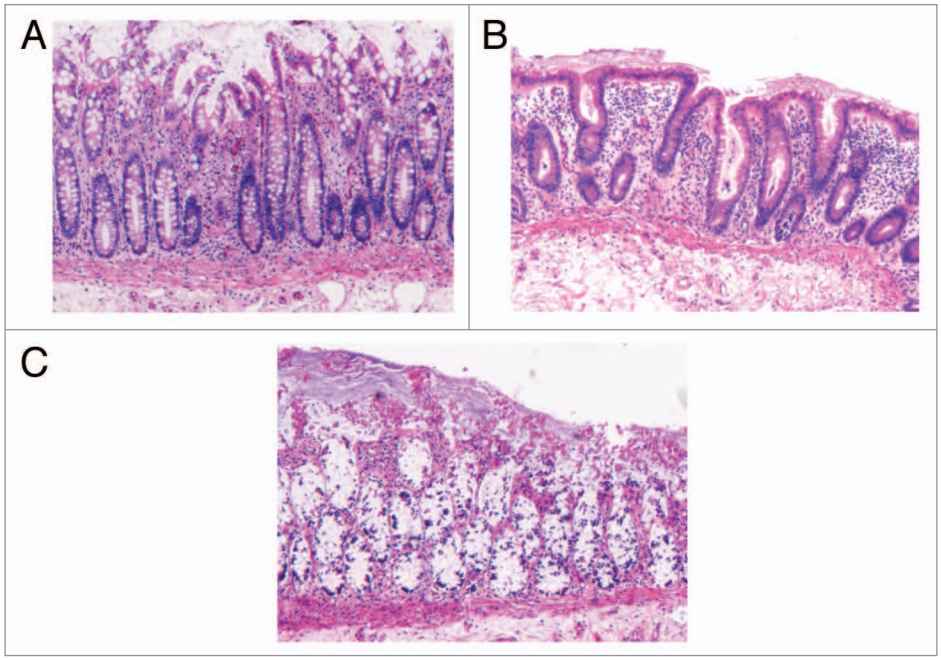

Figure 1. 24 hour long culture is only successful in O_2. (A) Tissue fixed immediately upon arrival in the laboratory; (B) Tissue cultured for 24 hours in an atmosphere of 99% O_2 in the pressure of 1 Atm; C. Tissue cultured for 24 hours in a conventional incubator. Original magnification: 5x (panels modified from Tsilingiri et al. Gut, 2012).

including attachment/degradation of mucus, competition with the commensal microbiota and resistance to antimicrobial peptides produced by the epithelial barrier.[8] As this kind of interactions is impossible to replicate on a cell line, it would follow that the best alternative would be to test treatments of interest directly on intact intestinal mucosa. Organ cultures of the intestinal mucosa have been set up many decades ago, and various protocols have been examined in order to come up with a process that would correctly maintain tissue viability in the long-term. Polarity is very important for the intestinal epithelial layer's proper function as the same stimulus can elicit completely different immune responses when applied apically or basolaterally.[9-11] Polarized stimulation and long-term analysis of the effects, however, has been quite a tricky task for researchers and not many approaches so far have satisfied those criteria.

We recently presented the model we devised to achieve polarized challenge. This was done by gluing a cave cylinder on the apical face of the mucosa using surgical glue, whose presence on the tissue was not detrimental (**Fig. 2**). We observed that once the conditions for stimulation and culture were standardized, results were quite reproducible and statistical significance was achieved even with a relatively low number of samples. We used our model to test three different Lactobacilli strains and succeeded in showing different effects. Here, we also show diverse effects of basolateral stimulation between a pathogenic agent (Salmonella) and the most anti-inflammatory of our probiotic strains.

We have managed to optimize a physiologically relevant model for the testing of any kind of treatments directly on intestinal mucosa, which we hope will become a routine complementary approach for obtaining robust pre-clinical data.

Setup of the Model and Viability Assessment

As the need for more physiological models of the human intestine on which to test various kinds of treatment has long been recognized, many attempts have been made in the past to keep tissue in culture in the presence of various stimuli and try to monitor the explants' immune response. However, until quite recently, researchers had been indiscriminately keeping human or animal explants in conventional incubators or in 100% O_2 atmosphere,[12-15] thus the first thing we wished to assess was the viability of the tissue in conventional incubators. We found that even in the absence of the cylinder, the tissue was only viable when incubated in 100% O_2 (**Fig. 1**). Indeed, if incubated in 100% O_2, the tissue as well as the commensals remained viable for up to 36 h as attested by fluorescence in situ hybridization (FISH) (**Fig. 2D**), though by that time patches of apoptotic epithelium started to show.[16] Moreover, in 100% O_2 the presence of the cylinder (**Fig. 2D**) and the surgical glue did not appear to be detrimental to the tissue's wellbeing, as the amount of LDH secreted by the explant in the supernatant did not increase (**Fig. 2C**, stimulation with Salmonella was used as a positive control in this case).

As mentioned, it has been shown and is well established that properly polarized (apical) stimulation of the intestinal epithelial layer is key for a proper homeostasis and the induction of tolerogenicity to innocuous antigens.[10] We demonstrated that in this set-up, challenge in the absence of the cylinder, led to stimulation via a non-physiological basolateral route occurring from the cut sides of the explant, as attested by bacteria localization and exaggerated cytokine secretion.[16]

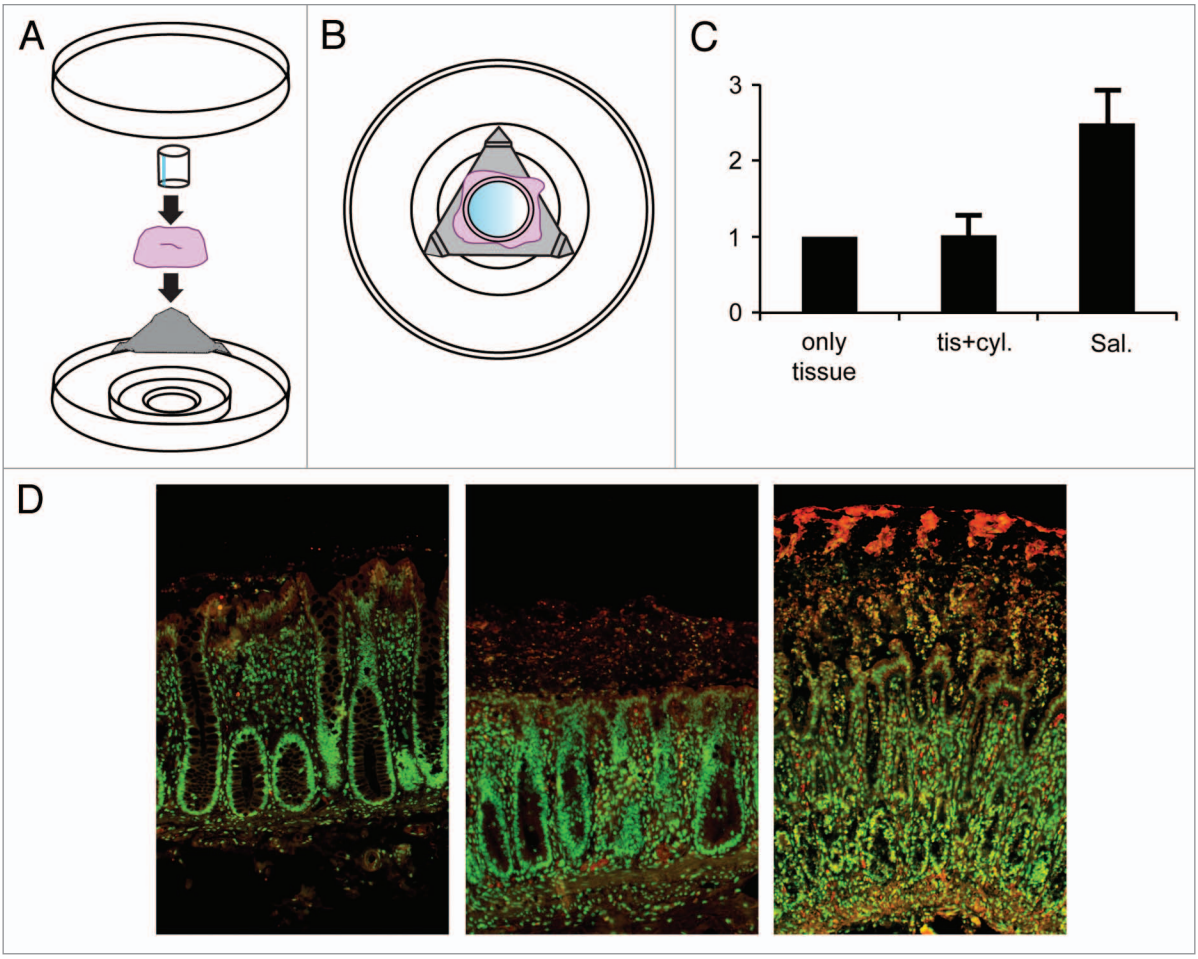

Figure 2. The presence of the surgical glue and the cylinder does not induce significant apoptosis on the explant (A) and (B). Schematic setup and mounting of the explant with the cylinder; (C) The presence of the cylinder does not lead to increased LDH secretion; (D) FISH: both the tissue and the commensals are viable for up to 36 hours. Green, nuclei; DAPI red, bacteria. Left panel, tissue fixed upon arrival in the laboratory; middle panel, tissue cultured for 24 hours; right panel, tissue cultured for 36 hours, original magnification: 10×, middle panel modified from Tsilingiri et al. Gut 2012.

Further, we show here that basolateral challenge elicits different results according to the stimulus. Challenge with Salmonella FB62 in the absence of the cylinder leads to extensive crypt apoptosis and NfκB translocation to the nucleus. However, if a truly benign probiotic strain, such as *L. paracasei* is used in the same way, this is not the case (**Fig. 3**) and the observed NfκB activation is largely similar to that of the uncultured sample. Finally, as a proof of concept experiment we also used the cylinder for the opposite purpose, namely to see whether the area of the explant "enclosed" in the cylinder would remain protected from a potential non-polarized stimulation with Salmonella. Indeed, as shown in **Figure 4**, the cylinder not only sufficiently maintains stimuli in the inside, but also on the outside.

Probiotic Activity in Health and Disease

The potential anti-inflammatory activity of certain probiotic strains has long been praised. These positive comments have so far been based largely in speculation after preliminary data, and feedback from healthy consumers with only minor intestinal problems. However, an important lesson from studying different probiotics is that even within the same genus, Lactobacilli for example, different strains may have different activities. Hence simple, easily reproducible and physiological enough models are needed for the separate testing of every strain before eventual introduction to the food industry or clinical practice. Having modified classical ex vivo organ culture protocols in the way described above, we succeeded in showing that three strains, all of them belonging to the Lactobacillus genus, could actually have quite different effects on healthy intestinal mucosa.[16] These results confirmed previous work performed in our laboratory, where differential effect of said probiotics was also shown on complex co-culture models as well as a mouse model of colitis.[17] On that occasion, we had established a protective effect for *Lactobacillus paracasei* B21060, as mice pre-treated with that strain showed a significantly reduced disease index during DSS colitis, whereas the other two Lactobacilli strains were by contrast detrimental and even resulted in an increased mortality rate. Thus, we had high hopes for applying *L. paracasei* B21060 on extensively inflamed IBD mucosa. To our surprise, however, live

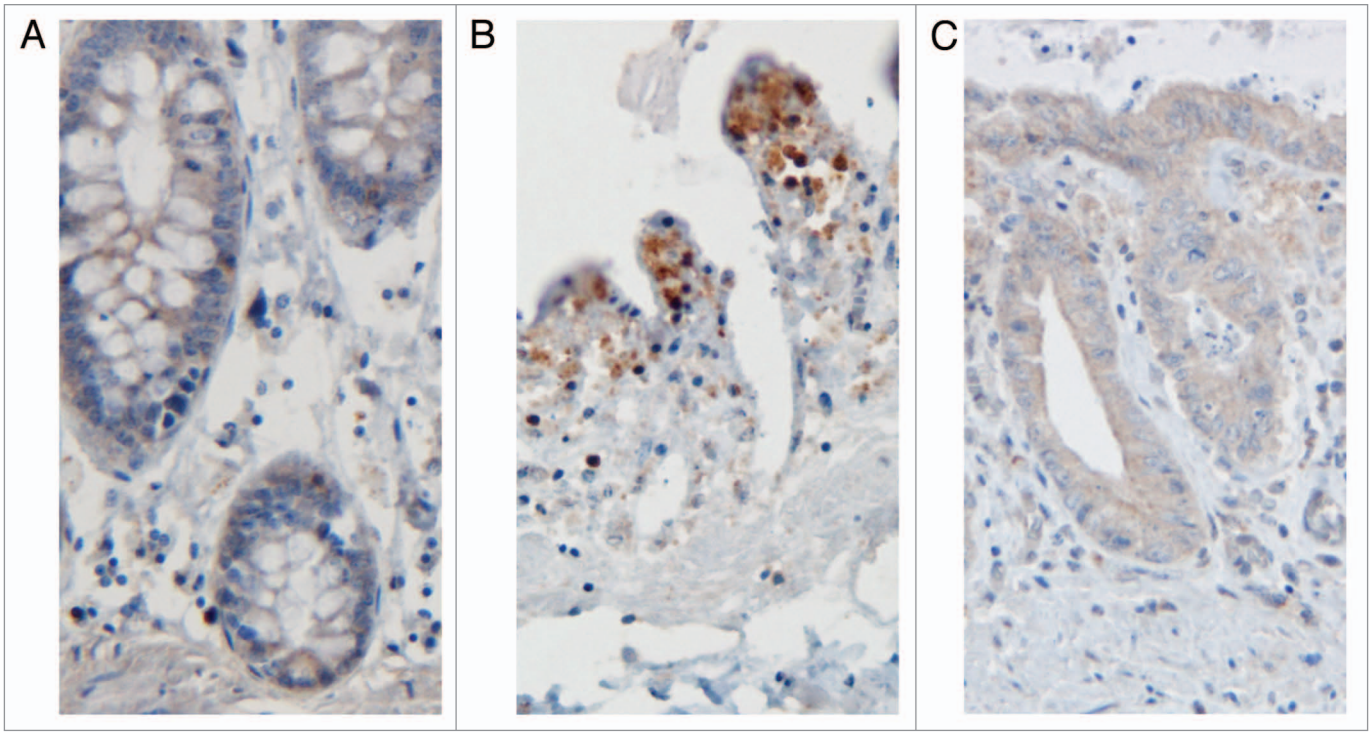

Figure 3. Non-polarized stimulation with a probiotic does not lead to extensive apoptosis or NfκB activation like Salmonella does—staining for p65 (A) Untreated tissue; (B) Tissue stimulated with Salmonella in the absence of the cylinder; (C) Tissue stimulated with *L. paracasei* in the absence of the cylinder.

bacteria of all three strains proved detrimental for patients' mucosa, with ileal Crohn's tissue being particularly sensitive to this kind of treatment. Noticeably, this would indicate that preventive strains are not necessarily going to be adequate for induction of remission as well, and hence preclinical data for every strain should be obtained in an experimental setting that is as similar as possible to the clinical situation for which the strain will be used.

The Potential of Postbiotics

One of the most important alleged properties of probiotic strains is the "shield effect" they might provide in a healthy intestine against potential pathogens. This "shield effect" could be the result of a variety of actions of beneficial microbes; in some cases the bacteria could act on the epithelial cells to make them more resistant to invasive strains, for example by upregulating production of tight junction proteins[18] or antibacterial molecules,[19] or conserving the mucus layer.[20] Alternatively, the proliferation of potentially dangerous strains could be inhibited by probiotic strains merely because of the niche competition phenomenon. Of note, mediation of one of these mechanisms of action does not necessarily exclude the possibility of the others being true at the same time. In some cases, these beneficial actions of the probiotics could be due to structural components of the latter interacting with eukaryotic cells components or products, or they could be mediated by certain metabolites produced in situ by the bacteria.

Indeed, we had previously observed that the anti-inflammatory action of the *Lactobacillus paracasei* B21060 strain was due to a soluble mediator to be found in the culture supernatant (henceforth SN) of this particular strain; pretreatment with SN, even at very low concentrations, was very efficient in inhibiting the pro-inflammatory effects of Salmonella on monocyte-derived dendritic cells, whereas live bacteria from which the SN had been extensively washed off before use lost this anti-inflammatory capacity (ref. 17 and unpublished data). Using our newly developed model, we not only confirmed that this action of the SN was evident ex vivo, but we also showed that in this case, rather than an effect of the SN on Salmonella viability and/or proliferation, a direct effect on the intestinal epithelial layer was the reason why Salmonella could not penetrate the tissue when the latter had been "conditioned" with SN prior to challenge. Microarray experiments on stimulated explants will show which pathways are modified after SN treatment and will shed light on the molecular mechanisms of this particular interaction.

Postbiotics as Therapeutic Agents for IBD

We previously described that even live probiotic bacteria that are completely innocuous on healthy tissue even when applied basolaterally can be detrimental for IBD tissue. Thus we reasoned that if the anti-inflammatory potential of one of these strains lay in the SN of the culture, that SN could potentially be used as a safer alternative for IBD treatment, eliminating the need and risk of using live bacteria. Besides, it had been previously described that the anti-inflammatory effect of

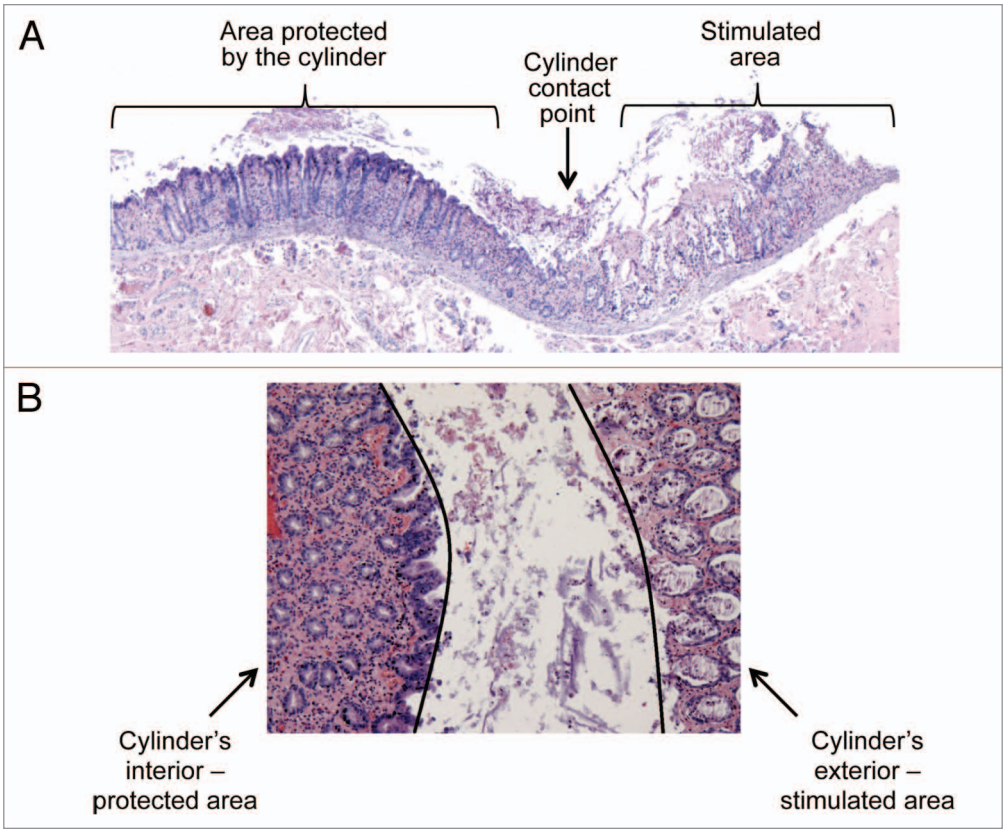

Figure 4. The cylinder does not only keep the stimulus inside, but potentially also outside (A) transverse section of explant stimulated with Salmonella outside of the cylinder, original magnification: 2×; (B) vertical section of the same explant, black lines: cylinder border, original magnification: 4×.

certain beneficial bacteria was associated with cellular metabolic products.[21]

We treated IBD explants with SN and observed that it was quite effective in downregulating a variety of proinflammatory cytokines at a low concentration (5%) which allowed us to maintain the culture medium's usual pH. Also in the case of IBD tissues, the SN not only improved tissue wellbeing in general, but, once again, it was capable of preventing Salmonella invasion (**Fig. 5**).

These data would indicate that even though certain strains might be adequate for prolonging remission periods, it would still not be wise to use them during active disease, at least not without diligently testing them first in a similar context. On the contrary, a potently anti-inflammatory postbiotic might be a safer alternative to live bacteria for IBD patients.

Discussion

The importance of a properly balanced gut flora for the maintenance of homeostasis and wellbeing has emerged in the past decade. Probiotics are, by definition, live microorganisms which, when administered in adequate amounts confer specific health benefits to the host. Indeed, probiotics have been widely used in recent years to alleviate minor to moderate intestinal discomforts, such as occasional or chronic constipation, diarrhea, IBS, etc. and it is estimated that the probiotic market moves around 30 billion US$ globally every year. In the clinics, probiotics have been successfully used for the prevention or cure of certain inflammatory conditions such as mastitis or atopic dermatitis, and interestingly, studies show that they could be used to lower health care costs, as patients receiving probiotics or synbiotics pre- or postoperatively were shown to spend significantly less time in intensive care and have a significantly reduced chance of post-operative infections and complications.[22-24] After such encouraging results however, we do run the risk of probiotics being viewed as a panacea for any possible inflammatory disorder. This could lead to the use of inadequately tested strains, or combination of strains, in irrelevant conditions. The results of such clinical trials designed in haste with insufficient pre-clinical data[4] have triggered a fervent debate on how safe it would be to actually use live bacteria in acute inflammation, and it has even led to European Food Safety Authority (EFSA) passing very strict laws against health claims made on any kind of food industry product.

Thus, the potential utility of valid models like the one described here for obtaining robust pre-clinical data becomes apparent. Even though cell co-culture and mouse models used so far have been invaluable for obtaining mechanistic insight on host-microbe interactions, it is admittedly much less plausible to evaluate the human bowel's immune response to any stimuli using those models. Hence, we feel that this model could be a reproducible and simple, yet not simplistic complementary approach for the testing of any kind of treatment that requires polarized application on human intestinal mucosa.

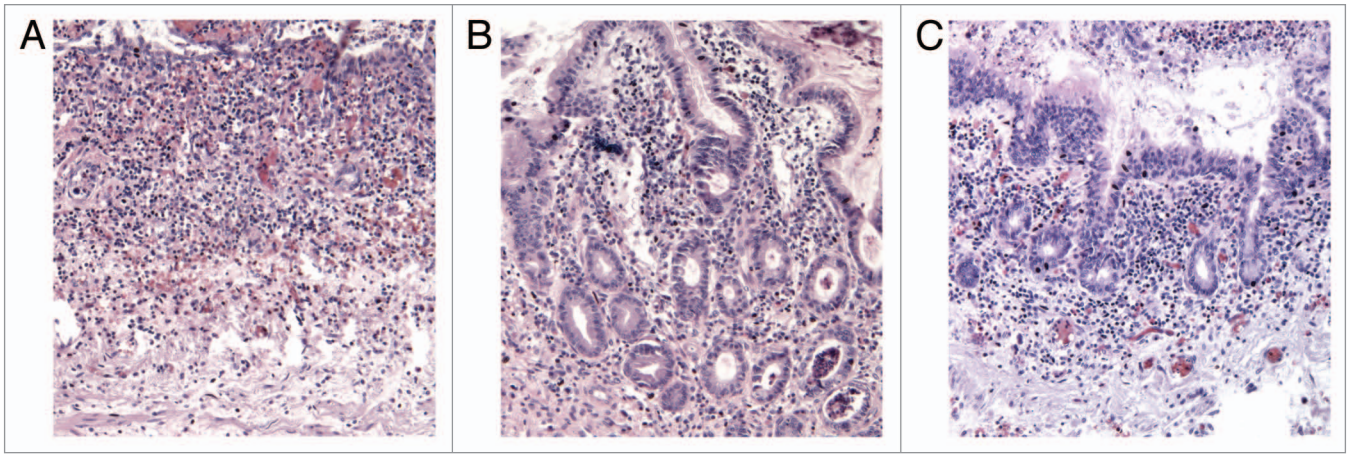

Figure 5. *L. paracasei* supernatant (SN) impedes Salmonella entrance and maintains tissue architechture and wellbeing on IBD tissues as well. (A) Colonic Crohn disease tissue stimulated with Salmonella; (B) The same tissue incubated with SN alone; (C) The same tissue challenged with Salmonella in the presence of 5% SN; original magnification: 10×.

Still, the feasibility of a large number of experiments on this model actually mainly depends on sample availability and influx, as we have so far strictly used surgical specimens not necessary for diagnosis. Thus, this system is currently not the best option for high throughput screening of a large number of strains/treatments. Further, as the maximum culture time so far has not exceeded 36 h, it becomes difficult to adapt more complicated assays to this model, like siRNA protocols which could come in very handy if molecular mechanisms were to be studied on this set-up. We are currently working to optimize culture in the presence of the cylinder for longer time frames in order to try out relevant assays on this model.

Nevertheless, the system we have described undoubtedly provides the researcher with a good amount of material for many different assays once testing has been performed (microarray, qPCR, western blotting, cytokine profiling, IHC/IF, etc.) When other types of assays, such as functional characterization of lamina propria cell components after stimulation and culture, are optimized, this set-up will provide various potential read-outs and the possibility to confirm any data obtained with more than one analysis method. Thus, it will become an asset not only for the application of potential treatments and evaluation of the tissue's immune response, but also for the study of interactions between the mucosa and pathogenic strains, helping to further elucidate key players involved in infection processes,[25] as well as the possibility of using bacterial strains as vehicles of vaccine delivery via the mucosal route, a subject on which much debate has arisen in recent years.[26]

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