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RESEARCH ARTICLE

The Epithelial Barrier Model Shows That the Properties of VSL#3 Depend from Where it is Manufactured

Paola Palumbo*, Francesca Lombardi, Maria Grazia Cifone and Benedetta Cinque

Department of Life, Health & Environmental Sciences, University of L'Aquila – Building Delta 6, Coppito, L'Aquila, Italy

Abstract: ***Background:*** VSL#3 has been extensively investigated and is currently recommended for the prevention and treatment of chronic pouchitis and ulcerative colitis. Nonetheless, *in vitro* and *in vivo* studies have recently shown variability in the VSL#3 efficacy often attributed to the manufacturing process.

***Objective*:** The aim was to comparatively study the *in vitro* effects of two VSL#3 preparations produced in different sites (named US- and Italy-made VSL#3) on CaCo-2 epithelial barrier model in terms of trans-epithelial electrical resistance (TEER), dextran flux and expression of Tight Junctions (TJ) proteins *i.e.* zonulin-1 (ZO-1) and occludin, in the absence or presence of a heat stress-related damage of monolayer.

***Methods*:** TEER was evaluated on CaCo-2 differentiated monolayers. Epithelial permeability of polarized monolayers was assessed by measuring the FITC-labeled dextran flux from the apical to basolateral chambers. ZO-1/occludin levels were analyzed by western blot analysis. A set of experiments was performed to compare the effects of both VSL#3 on TEER values, dextran flux and ZO-1/occludin expression in CaCo-2 monolayers after heat stress exposure.

***Results*:** US- and Italy-made VSL#3 have opposing effects on TEER values, dextran flux, and ZO-1/occludin expression, being all these parameters negatively influenced just by Italy-made product. US-made probiotic did not affect baseline TEER, dextran flux and ZO-1 expression and strongly increased occludin levels. Of note, pre-treatment of monolayer with US-made VSL#3, but not Italy-made product, totally prevented the heat-induced epithelial barrier integrity loss.

Conclusion: Our data trigger the need for reassessing efficacy or safety of the Italy-made VSL#3 considering intestinal epithelial barrier plays an important role in maintaining host health.

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1. INTRODUCTION

Variability in probiotics manufacturing may affect their properties, with potential implications on their efficacy and safety. Each step involved in the industrial production of the bacterial strains, i.e. growth conditions, culture media, cryoprotectants, food formulation, processing, storage conditions, may affect the probiotic properties of the bacteria [1-6]. Strain identity and manufacturing should be consistent over the years to assert the legacy of a certain probiotic product commercialized and known under a certain trademark.

VSL#3, a mixture of 8 different bacterial strains has been extensively investigated and is currently recommended for the prevention and treatment of chronic pouchitis and ulcerative colitis [7-9]. Nonetheless, *in vitro* and *in vivo* studies have recently shown a surprising variability in the VSL#3 efficacy and safety [10-13]. Since available promotional information regarding VSL#3 confirms the legacy of the product by referring to the "*over 140 studies and reviews having been conducted and published since the start of its history"*, the only viable hypothesis behind the loss of the protective effects of the "new" VSL#3 is that the change of the manufacturing site affected the biological characteristics of the product.

Our group has already reported some of the results obtained comparing the US-made VSL#3 versus the Italy-made VSL#3. The two products, even though sold under the same brand VSL#3, differently influence *in vitro* tumor cell lines as well as repair process of scratched intestinal epithelial cell monolayer [10, 11]. Since a recent paper using animal models of IBD has reported that the "new" Italy-made formulation failed to reduce gut inflammation and worsened intesti-

*Address correspondence to this author at the Department of Life, Health & Environmental Sciences, University of L'Aquila, Building Delta 6, Coppito, 67100 L'Aquila, Italy; Tel: +39-0862-433554;
E-mail: paola.palumbo@univaq.it

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nal permeability, it occurred to us that our data on CaCo-2 epithelial barrier model, here reported, may contribute to explain what observed in the animals and *ex vivo* in humans [12, 13].

Human colon cancer cells (CaCo-2) are widely used as an optimum *in vitro* model for studies on intestinal barrier functions [14-16] due to their ability to spontaneously differentiate into a polarized monolayer when grown on porous inserts. Under these conditions, CaCo-2 close up and tighten becoming similar to the intestinal barrier composed by enterocytes with microvilli (brush border) on the apical side and a basolateral side, and express TJ proteins, similar to the small intestine [17, 18]. Our data confirm that probiotic formulations can be analyzed on trans-epithelial electrical resistance (TEER), dextran flux and expression TJ proteins *i.e.* zonulin-1 (ZO-1) and occludin, in the absence or presence of a heat stress-related damage of cell monolayer, to detect *in vitro* functional differences which are clinically relevant.

2. MATERIALS AND METHODS

2.1. US-made and Italy-made VSL#3

Some boxes of VSL#3® distributed by Ferring Pharmaceuticals were purchased in the UK (lot number 507132, expiry date 07/2017; Italy-made VSL#3) for testing and comparison with the original product distributed by Ferring in Italy (lot number DM538 expiry date 12/2017; US-made VSL#3). According to the information on the boxes, each 4.4 g sachet provides a blend of 450 billion bacteria. As yet reported [10], the strains composing each product are under different names. The US-made formulation contains in certain proportions the strains*: Streptococcus thermophilus* DSM24731, bifidobacteria (*B. longum* DSM24736, *B. breve* DSM24732, *B. infantis* DSM24737), lactobacilli (*L. acidophilus* DSM24735, *L. plantarum* DSM24730, *L. paracasei* DSM24733, *L. debrueckii* subsp. *bulgaricus* DSM24734), while the Italy-made product contains: *Streptococcus thermophilus* BT01, bifidobacteria (*B. breve* BB02, *B. longum* BL03, *B. infantis* BI04), lactobacilli (*L. acidophilus* BA05, *L. plantarum* BP06, *L. paracasei* BP07, *L. debrueckii* subsp. *bulgaricus* BD08). Since the two formulations are commercialized under the same brand VSL#3, we assumed that the two formulations are genetically identical. The products were always handled according to the manufacturer's instructions and the sachets once opened were utilized immediately and then discarded.

2.2. Preparation of Bacterial Samples

Stocks of 1 g of each US-made or Italy-made formulation were suspended in 10 ml of DMEM supplemented with 1% non-essential amino acid, 1 mM sodium pyruvate and 2 mM L-glutamine (Euro Clone, West York, UK). For the cell treatment, bacterial final concentration of 10^8 CFU/ml was used.

2.3. CaCo-2 Cell Culture

The human colon adenocarcinoma cell line, CaCo-2 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and cultured as previously described [19]. Briefly, cells were grown in the DMEM supplemented with 10% (v/v) FBS, 1% (v/v) non-essential amino acid, 1 mM sodium pyruvate and 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium), in a humidified incubator 5% CO_2, 95% air atmosphere at 37°C. The cell culture medium was replaced every 2 days until cells reached 60–70% confluence and then harvested using a trypsin-EDTA solution to detach them from the bottom of the flask. CaCo-2 cells with a passage number of 18 to 20 were used for all experiments. Cell viability was analyzed by the trypan blue exclusion staining (0.04% final concentration for 5 minutes). Cells were counted in a Bürker chamber by microscopy (Eclipse 50i, Nikon Corporation, Japan).

2.4. Trans-Epithelial Electrical Resistance (TEER)

Epithelial permeability was evaluated by Trans-Epithelial Electrical Resistance (TEER) using a Millicell® ERS-2 (Electrical Resistance System, Millipore, MA, USA) equipped with STX01 electrode (World Precision Instruments, Sarasota, FL) on CaCo-2 differentiated monolayers. CaCo-2 cells were seeded at a density of $4.5x10^5$ cells/cm^2 on polycarbonate inserts in 12 well plates transwell chambers (12 mm diameter, 0.4 µm pore size) (Falcon, One Riverfront Plaza, NY, USA). Each insert was placed in a well in a 12-well plate with 1 ml of medium in the apical chamber and 1,5 ml medium in the basolateral chamber. Differentiated CaCo-2 monolayers were used at 18-21 days old. The CaCo-2 monolayers were prepared the day before the TEER assay by removing the media and adding DMEM with 1% non-essential amino acids (without FBS and antibiotics) to ensure no damage to the bacterial cells. Wells containing only medium were used as blank controls. TEER measurements of each well have been registered at different incubation times (0-6 h) with or without addition of probiotic products at 10^8 CFU/ml as final concentration. No significant influence on cell count and viability was observed after treatment with both VSL#3 formulations (not shown). A set of experiments was performed to also compare the effects of a pretreatment of CaCo-2 monolayer for 4 hours with the VSL#3 mixtures at 10^8 CFU/ml on TEER impairment induced by heat stress exposure (40°C for 10 minutes).

The CaCo-2 monolayer TEER values were calculated subtracting the blank resistance (background electrical resistance from an insert without cells including filter and medium) and multiplying the result by the effective growth area of the membrane (1.12 cm^2). TEER values at >500 Ω/cm^2 indicated the successful establishment of the *in vitro* intestinal epithelial barrier model. Results are expressed as a percentage of initial value (baseline).

2.5. Paracellular Permeability Assay

Epithelial permeability across polarized CaCo-2 monolayers was assessed by measuring the flux of the fluorescein isothiocyanate (FITC)-labeled dextran FD4 (1 mg/ml) (Sigma Chemical Co., St. Louis, MO, USA) from the apical chambers to basolateral chambers. FD4 solution was added to the apical chamber, for 2 hours at 37°C in sterile conditions. Fluorescence intensity in the basolateral compartment was measured by fluorometer (Perkin Helmer Victor X4) at excitation and emission wavelength of 492 nm and 520 nm, respectively.

Known concentrations of FITC-dextran FD4 were used to perform a standard curve obtained by serial dilutions of FD4. A set of experiments was performed to also compare the effects of a pretreatment of CaCo-2 monolayer for 4 hours with the VSL#3 mixtures at 10^8 CFU/ml on dextran flux increase induced by heat stress exposure (40°C for 10 minutes).

2.6. Western Blot Analysis

CaCo-2 pellets were collected after incubation with or without US-made and Italy-made VSL#3 and homogenized in ice-cold RIPA buffer (phosphate buffer saline pH 7.4 supplemented with 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, 5 mM EDTA, 100 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM PMSF, 2 mM ortovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin) (Sigma Chemical Co., St. Louis, MO, USA). A set of experiments was performed to also compare the effects of a pretreatment of CaCo-2 monolayer for 4 hours with the VSL#3 mixtures at 10^8 CFU/ml on ZO-1 and occludin expression levels in heat-stressed CaCo-2 monolayers (40°C for 10 minutes). Homogenates were centrifuged at 600*xg* for 20 min at 4°C and the supernatants were quantified for protein content using the BCA protein assay kit (Pierce, Rockford, USA). Samples (25 µg/lane) were run on 12% SDS-polyacrylamide gels according to standard procedures, and proteins were transferred onto nitrocellulose membranes. Non-specific binding sites were blocked with 5% not fat dry milk for 1 hour at room temperature and membranes were incubated overnight at 4°C with primary antibodies anti-occludin, anti-ZO-1 and anti-GAPDH (Origene, 9620 Medical Center Drive Suite 200 Rockville, MD, USA). As secondary antibody an anti-rabbit IgG (for occludin and ZO-1 reactivity detection) and an anti-mouse IgG (Millipore, Burlington, Massachusetts, United States) for GAPDH reactivity detection were used, respectively. Immunoreactive bands were visualized by ECL chemiluminescent substrate reagent according to the manufacturer instructions and acquired by UVItec Alliance (Cambridge UK). Densitometric analysis was performed by software provided by the company. Relative ZO-1 and occludin band intensities were normalized to relative GAPDH bands.

2.7. Statistical Analysis

Data were reported as mean ± SEM of two or three independent experiments routinely performed in duplicate as indicated in each figure. Data were analyzed using Prism 6.0 GraphPad Software, San Diego, Ca. Statistical significance was determined using a one-way or repeated measures two-way ANOVA followed by Bonferroni post-hoc test. Differences were considered significant when $P<0.05$.

3. RESULTS

3.1. Italy-made but not US-made VSL#3 Impaired TEER of CaCo-2 Monolayer

In order to evaluate the ability of the different lots of VSL#3 to influence the epithelial integrity, TEER measurements were registered at different time intervals (0, 1, 3, and 6 h) on CaCo-2 untreated or treated with each probiotic mixture at 10^8 CFU/ml (final concentration). Results reported in Fig. (**1A**) show the TEER values expressed as percentage change from baseline and mean ± SEM of two independent experiments performed in duplicate. While the US-made product, despite a slight increasing trend, did not affect significantly the values of TEER compared to control at all time intervals, the Italy-made VSL#3 induced a time-dependent TEER reduction when compared to both untreated and US-made probiotic treated cells which was statistically significant at 3 and 6 h incubation (*P<0.01* at 3 h and *P<0.001* at 6 h vs untreated; *P<0.001* at 3 h and *P<0.0001* at 6 h *vs* US-made VSL#3). In Fig. (**1B**) representative microscopy images from transwell chambers at 20X magnification with CaCo-2 monolayers untreated or treated with US- or Italy-made VSL#3 at 10^8 CFU/ml for 3 h are shown. In accordance with TEER results, the appearance of cell-free areas (holes) is evident in the monolayer treated with Italy-made VSL#3, thus suggesting a damage to epithelial barrier.

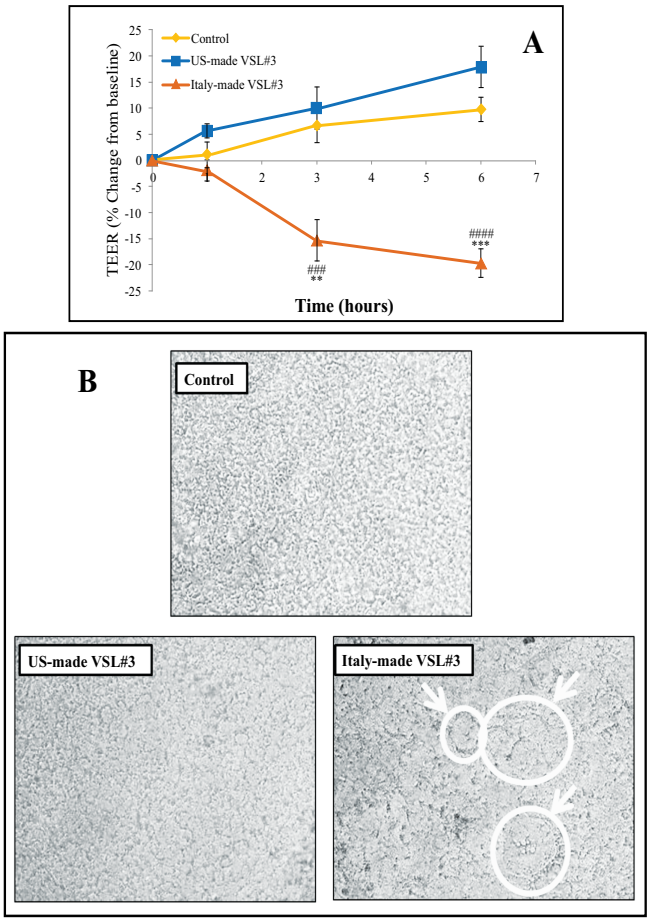

Fig. (1). Effects of US- and Italy-made VSL#3 on TEER levels. (**A**) TEER values expressed as % change of baseline over time (0-6 hours) in the presence or absence of US- or Italy-made VSL#3 at 10^8 CFU/ml. Data are expressed as mean ± SEM of two independent experiments performed in duplicate. Differences among mean values were assessed by two-way repeated measures ANOVA following by Bonferroni post-hoc test (**$P<0.01$ and ***$P<0.001$ *vs* control; ###$P<0.001$ and ####$P<0.0001$ *vs* US-made VSL#3). (**B**) Representative microscopy images from transwell chambers at 20X magnification with CaCo-2 monolayers untreated (control) or treated with US- or Italy-made VSL#3 at 10^8 CFU/ml for 3 h. Circles and arrows indicate the cell-free areas (holes).

3.2. Italy-made but not US-made VSL#3 Impaired Paracellular Permeability

To compare the ability of US-made and Italy-made VSL#3 formulations to influence paracellular permeability of differentiated CaCo-2 monolayers, the measurement of fluoresceinated dextran-4 amount through the monolayers was used. Flux of FITC-dextran-4 (FD4) in differentiated CaCo-2 cell monolayers treated or not with VSL#3 formulations for 3 hours was assayed as described in Materials and Methods' section. In agreement with the TEER values registered at 3 h treatment and reported in Fig. (**2A**), the 3 h-exposure to Italy-made VSL#3 led to a significant increase (~23-30%) in FD4 flux when compared either to control (*P<0.05*) and US-made VSL#3 (*P<0.05*), thus confirming a barrier damage. On the contrary, the addition of US-made VSL#3 formulation did not affect the FD4 flux when compared to untreated monolayer. The results expressed as µg/ml FITC-dextran recovered from basolateral compartments are shown in Fig. (**2B**) as means ± SEM of two independent experiments performed in duplicate.

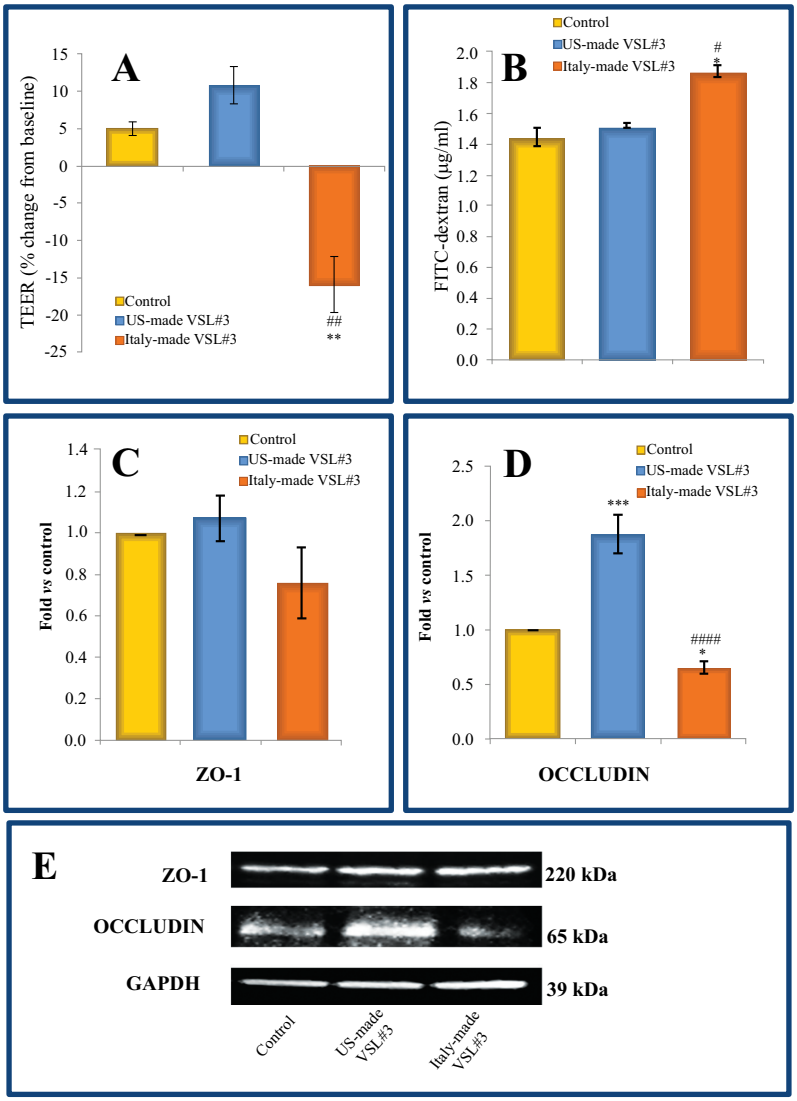

Fig. (2). Effects of US- and Italy-made VSL#3 on FD4 flux and TJ protein levels. (**A**) TEER values expressed as % change of baseline at 3 hours in the presence or absence of US- or Italy-made VSL#3 at 10^8 CFU/ml. Data are expressed as mean ± SEM of three independent experiments performed in duplicate. Differences among mean values were assessed by one-way ANOVA following by Bonferroni post-hoc test (***P<0.01 vs* Control; *##P<0.01 vs* US-made VSL#3). (**B**) FD4 flux across the differentiated CaCo-2 monolayers was measured after 3 h treatment in the presence or absence of US- or Italy-made VSL#3 at 10^8 CFU/ml. Results are expressed as mean ± SEM of two independent experiments performed in duplicate. Differences among mean values were assessed by one-way ANOVA following by Bonferroni post-hoc test (**P<0.05 vs* control; *#P<0.05 vs* US-made VSL#3). (**C**) and (**D**) Densitometric analysis of western blot bands respectively for ZO-1 and occludin levels in CaCo-2 monolayers incubated for 3 h in the presence or absence of US- or Italy-made VSL#3 at 10^8 CFU/ml. Values were normalized to GAPDH and the results are presented as fold increase *vs* control and represent mean ± SEM of three independent experiments in duplicate. Differences among mean values were assessed by one-way ANOVA following by Bonferroni post-hoc test (**P<0.05 vs* control; ****P<0.001 vs* control; *####P<0.0001 vs* US-made VSL#3). (**E**) Western blot images from one representative out of three independent experiments for ZO-1 and occludin protein levels are shown.

3.3. Effects of US- and Italy-made VSL#3 on ZO-1 and Occludin Levels

In order to further investigate the ability of both US- and Italy-made VLS#3 preparations to influence the levels of TJ proteins, ZO-1 and occludin analysis by western blotting was performed in CaCo-2 cells before and after 3 h treatment with bacterial samples. As shown in Fig. (**2C**), treatment with both VSL#3 preparations at 10^8 CFU/ml did not meaningfully alter the ZO-1 protein expression levels. On the other hand, the cells treated with US-made VSL#3 showed a significant increase of occludin level respect to control (*P<0.001*). Conversely, occludin expression in CaCo-2 after Italy-made VSL#3 exposure was significantly decreased as compared to both untreated (*P<0.05*) and US-made VSL#3 treated cells (*P<0.0001*) (Fig. **2D**). The results are expressed as mean ± SEM of three independent experiments in duplicate (ZO-1 or occludin expression, fold versus control). In Fig. (**2E**), images from one representative experiment for ZO-1 and occludin levels as analyzed by western blotting are shown.

3.4. US-made but not Italy-made VSL#3 Totally Prevents Heat-stress Induced Epithelial Barrier Damage

In order to compare the effects of VSL#3 formulations on heat damage induced in CaCo-2 cells, experiments on TEER measurements, paracellular dextran flux and ZO-1/occludin protein expression after exposure of cells to 40°C for 10 minutes have been performed. To this aim, cell monolayers were incubated with US- or Italy-made VSL#3 at 10^8 CFU/ml for 4 h before heat treatment. Results shown in Fig. (**3A**) are expressed as mean ± SEM of two independent experiments in duplicate. Heat stress induced in control monolayers a drop in TEER values with ~20-30% reduction when compared to relative baseline values. Of note, pre-treatment with US-made VSL#3 totally prevent the TEER heat-induced fall even inducing a slight increase in TEER value (*P<0.05 vs* heat-exposed control). By contrast, the Italian product was not able to protect against harmful heat damage being TEER measurements not significantly different when compared to control monolayers exposed to heat (*P>0.05*) and significantly lower than TEER values of US-made treated monolayers (*P<0.05*).

In accordance, heat stress-induced FD4 flux increase could be totally prevented just by pre-treatment with US-made VSL#3 but not Italy-made product (Fig. **3B**) (*P<0.01 vs* control and *vs* Italy-made treatment). Results are expressed as mean ± SEM of three independent experiments in duplicate.

The influence of pre-treatment with US- and Italy-made VSL#3 mixtures on the expression of ZO-1/occludin in CaCo-2 cells exposed to heat-induced stress was also investigated. Results are shown in Figs. (**3C** and **3D**) and expressed as mean ± SEM of three independent experiments in duplicate. Representative western blot images are also reported. Heating of cells for 10 minutes at 40°C induced a significant reduction of ZO-1 level (*P<0.05 vs* non-exposed untreated) (Fig. **3C**). As previously reported [20], no significant change was instead observed in occludin level between unexposed controls and cell monolayers exposed to heating for a short period. Pretreatment with US-made VSL#3 for 4 hours at doses of 10^8 CFU/ml fully preserved ZO-1 expression levels which were similar to those detected in untreated unexposed cells and significantly higher when compared to heat-stressed control and Italy-made probiotic (*P<0.01)*. In accordance with the results reported in Fig. (**2D**), also in these conditions US-made VSL#3 was able to strongly elevate occludin levels which resulted significantly higher either *versus* unexposed and heat-exposed controls or Italy-made treated monolayers (*P<0.05*, *P<0.05*, and *P<0.01*, respectively) (Fig. **3D**). Conversely, no significant difference was observed either for ZO-1 or occludin levels between Italy-made VSL#3 formulation-treated samples and heat-stressed controls (Fig. **3D**), thus suggesting that Italy-made formulation, also in this case, was not able to prevent the heat-induced drop of ZO-1 and occludin expression levels.

4. DISCUSSION

Genetically identical bacterial strains if produced differently do not have the same probiotic properties. For example, significant changes in the *in vitro* properties of early *L. rhamnosus GG* strains were reported, when funtional tests were done on fifteen different *Lactobacillus rhamnosus GG* isolates with 100% sequence homology [21]. This fact, attributed to different production processes and a long-term series of reinoculations, has also been described for *L. acidophilus* to such an extent that the outcome of the human intervention studies may be different [22]. Recently, the influence of technological treatments on the functionality of *Bifidobacterium lactis INL1* has been confirmed [5] and the antifungal activity of *Lactobacillus pentosus ŁOCK 0979* has been shown to be dependent from the presence of polyols and galactosyl-polyols in the culture medium [23]. Therefore, it is evident that genomic characterization of probiotic strains is not an appropriate solution to establish the consistency of a probiotic production.

Functional studies are needed to assess if production changes have a substantive impact on probiotic efficacy and safety. The marketer should do this task before the "new" product is introduced to replace the "old" product. For investigators, this is not an easy task since it is possible to make functional comparative studies only for the short period of time when both formulations – the "old" and the "new" - are available in the market. Once the "old" formulation passed its expiry date de facto there is a limitation of the tests that investigators can perform.

In the case of the VSL#3 product, Biagioli recently [12] failed to confirm that the Italy-made VSL#3 is comparable to the US-made VSL#3 for its protective action at the level of TJ [24-26], in terms of reduction of colitis [27-29] and colonic inflammation [30] in dextran sodium sulphate-induced (DDS) treated mice and in Muc2 mucin-deficient mice.

Our data here reported offer a molecular explanation for the discrepancy of the above-mentioned results. We were able to investigate both active and passive transport *in vitro* through the use the CaCo-2 cell line that is derived from a human colorectal adenocarcinoma. These cells maintained in culture differentiate into polarized enterocytes and form a monolayer of columnar cells that are coupled together by tight junction proteins that restrict the diffusion of substances across the barrier. Usually, the CaCo-2 cells are cultured on

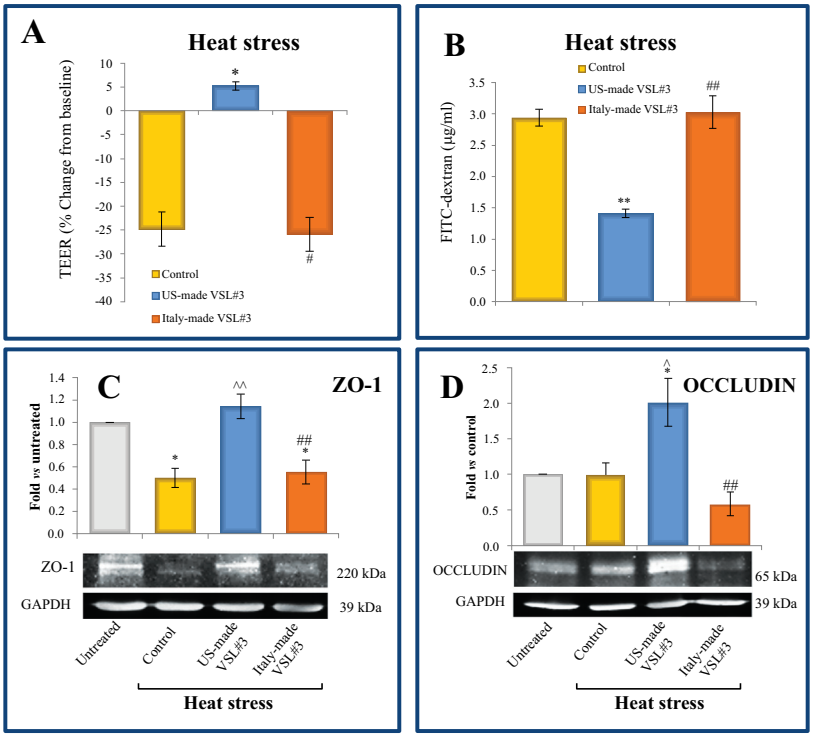

Fig. (3). Effects of pretreatment with US-made and Italy-made VSL#3 on TEER values, FD4 flux, and TJ protein levels in heat-stressed CaCo-2 differentiated monolayers. The effects of a 4 h pretreatment with US- or Italy-made VSL#3 at 10^8 CFU/ml before heat stress at 40°C for 10 min are shown. (**A**) TEER values expressed as % change of baseline at 3 h after exposure of CaCo-2 monolayers to 40°C for 10 minutes. Data are presented as means ± SEM of two experiments performed in duplicate. Differences among mean values were assessed by one-way ANOVA following by Bonferroni post-hoc test (**P<0.05 vs* control; *#P<0.05 vs* US-made VSL#3). (**B**) FD4 flux data are expressed as means ± SEM of three experiments performed in duplicate. Differences among mean values were assessed by one-way ANOVA following by Bonferroni post-hoc test (***P<0.01 vs* control; *##P<0.01 vs* US-made VSL#3). (**C**) and (**D**) Densitometry of bands from western blot analysis respectively of ZO-1 and occludin in CaCo-2 cells. Values were normalized to GAPDH. Histogram bars are relative to fold *vs* untreated cells. Results are expressed as mean values ± SEM of three independent experiments. Differences among mean values were assessed by one-way ANOVA following by Bonferroni post-hoc test (**P<0.05 vs* untreated; *^P<0.05* and *^^P<0.01 vs* heat-exposed control; *##P<0.01 vs* US-made VSL#3). Images from one representative out of three independent experiments for ZO-1 and occludin protein levels analyzed by western blotting are shown.

a semipermeable filter insert that defines a partition for upper and lower compartments. One electrode is placed in the upper compartment and the second one in the lower compartment, and the electrical resistance of the cellular monolayer is measured based on Ohm's law as the ratio of the voltage and current in ohms. The advantage of this method is it non-invasiveness and that can it permits an accurate quantitative measure of the barrier integrity. Using this approach we have generated evidence that US- and Italy-made VSL#3 have opposing effects on the CaCo-2 epithelial barrier model in terms of TEER values, paracellular dextran flux, and ZO-1 and occludin expression levels. Exclusively Italy-made VSL#3 negatively influenced all the above parameters. In fact, the US-made probiotic did not affect TEER values, dextran flux and ZO-1 expression and instead induced an increase of occludin levels. Of note, US-made VSL#3 totally prevented the heat-induced loss epithelial barrier integrity while the Italy-made product was not able to revert the wrecked epithelial barrier provoked by heating. Accordingly, US-made VSL#3 was also able to maintain the basal dextran flux and increased occludin expression also in the presence of heat stress. On the other hand, Italy-made VSL#3 was unable to avoid heat-induced increase of dextran flux and decrease of ZO-1 and occludin levels.

Assuming that available information on the composition of the Italy-made VSL#3 product is true, these major differences may be related to the higher ratio of dead bacteria present in the Italy-made VSL#3, as previously reported by us [10] or to the significantly higher concentrations of pyruvate, 1,3-dihydroxyacetone (DHA), uracil, and uridine found in the Italy-made product [13]. Interestingly, Biagioli *et al*. also showed evidence that challenging mice with 1,3-DHA increased intestinal permeability and inflammatory markers i.e. MCP-1 (monocyte chemotactic protein-1) and IL-8, meanwhile reducing ZO-1 and occludin mRNA levels in colon samples [12]. Trinchieri *et al*. found significant differences in DHA metabolism and production *in vitro*, between bacteria from US- and Italy-made VSL#3 preparations. Whereas US-made product bacteria were able to metabolize DHA, the bacteria present in the Italy-made VSL#3 produced DHA, which in turn induced a decrease of *S. thermophilus* viability, IEC-6 cell viability and repair rate of scratched IEC-6 monolayers [13].

Our data have a strong clinical implication since humans rely on an efficient intestinal barrier to protect themselves against pathogens and pathobionts while hosting beneficial bacteria. The intestinal mucosa consists of an epithelial layer, the lamina propria and the muscularis mucosa where

the epithelial layer is the guardian hurdle for nutrient absorption, drug permeation and toxic substances exclusion. The bioavailability of nutrients, drugs or other substances administered orally depends on intestinal barrier integrity that can be compromised in various diseases such as inflammatory bowel diseases (IBD), infectious enteritis, Necrotizing Enterocolitis (NEC), serious liver diseases [31-34].

5. LIMITATIONS OF OUR STUDY

Our explanation of the data is based on the public promotional information regarding the VSL#3, where there is no mention that the strains have changed, at the same time informing that the "new" Italy-made VSL#3 is now dairy free. Therefore, we elaborated that all the differences between the two formulations should be attributed to different manufacturing processes. However, recent publication Douillard *et al*. (2018) who received the individual strains from the VSL#3 manufacturer, but did not isolate from the finished product, indicated that the strains *Bifidobacterium longum subsp longum BL03* and *B. longum subsp. infantis BI04* presumed to be present in the Italy-made VSL#3 appeared to differ only by few single nucleotide polimorfisms (SNPs) [35]. In the supplementary Table **3** (https://doi.org/10.1371/journal.pone.0192452.s004) of the article of Douillard *et al*., it is reported that the best strain template for both BL03 and Bl04 strains from Italy made VSL#3 is the strain Bi-07. These findings strongly suggest that the Italy-made VSL#3 may have a different strain composition from the US-made VSL#3. The Authors relied on the information released by the producer regarding the legacy of the VSL#3 product, but the Douillard paper raises the concern.

CONCLUSION

The European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) advocated more stringent quality control of probiotics, and noted that "procedures such as fermentation, matrix composition, cell harvesting, spray drying, freeze-drying and storage conditions like temperature, humidity and pH, are just several out of a wider array of manufacturing determinants that can affect microbial survival, growth, viability and ultimately the study results and/or clinical outcomes" [36].

Probiotics are classed as food supplements or dietary and this means there is scant regulation of the manufacturing process and virtually no regulatory follow-up once a product is launched. Under current regulations, under a given brand name any probiotic formulation can be sold, even if it is significantly different from the formulation that was originally sold under that specific trademark.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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PP and FL carried out all the experiments and performed the statistical analysis of data; MGC and BC conceived the study; BC coordinated the definition of experimental protocols and the choice of methods to be used. All the Authors contributed to write the manuscript.

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