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Preclinical Evaluation of Tolerability of a Selective, Bacteriostatic, Locally Active Vaginal Formulation

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ABSTRACT

Background: Polybactum (Effik International, Brussels, Belgium) is a vaginal mucoadhesive product (medical device) designed to form a film that acts as a mechanical barrier with the aim of inhibiting colonization by specific pathogens. It contains polycarbophil, a bioadhesive agent, and lauryl glucoside (LG), a nonionic surfactant that reinforces the barrier effect through its tensioactive properties.
Objective: To assess the local safety profile, tolerability, and efficacy of Polybactum formulations.
Methods: The following studies were performed on 3 Polybactum formulations: 2 ovules (Type 1: LG 0.04% and Type 2: LG 0.1%) and 1 gel formulation. Bacteriologic tests assessing the effects on normal vaginal flora and pathogens; in vitro and in vivo tests designed to assess cytotoxicity, as well as irritant and sensitizing potentials; biocompatibility, barrier, residence time, and absorption tests using reconstituted human vaginal epithelium were performed.
Results: Polybactum is a selective bacteriostatic agent that is active against *Streptococcus agalactiae* and *Gardnerella vaginalis* while sparing normal vaginal flora; that is, *Lactobacillus* spp. It had no cytotoxic, irritant, and sensitizing effects nor did it impair barrier and fence functions of the vaginal epithelium. The Type 1 ovule showed film-forming properties in vitro. Finally, LG absorption through reconstituted human vaginal epithelium was negligible, ruling out the risk for possible systemic toxicity.
Conclusions: This favorable preclinical profile is encouraging and supports clinical studies on Polybactum Type 1 ovules for the prevention and management of recurring bacterial vaginosis.

Introduction

One of the most important discoveries in medicine during the past decade has been the diversity and extent of the human microbiome. The human body is now perceived as a superorganism in which a multitude of microbial genomes continually interact with the human genome. A growing body of evidence indicates that alterations of the microbiome are associated with a number of diseases.[1]

The vaginal microflora mainly consists of lactobacilli, which dominate over pathogenic anaerobes, contributing to vaginal health. Alterations in the vaginal microflora, called bacterial vaginosis, are very common, occurring in 20% to 50% of fertile, premenopausal, and pregnant women.[2] Bacterial vaginosis is associated with adverse pregnancy outcomes (ie, prematurity), increased risk of postpartum infections such as endometritis, and increased infectious complications after gynecologic procedures, as well as pelvic inflammatory disease. It is also a risk factor for urinary tract infections and for the acquisition of sexually transmitted diseases, and can directly affect fertility.[2–7] Standard therapy involves oral or local administration of metronidazole or intravaginal clindamycin. Although short-term success rates may be as high as 85%, medium- to long-term cure rates are low: the recurrence rate is up to 35% at 1 month, 50% at 3 months, and 70% at 12 months.[8]

Polybactum (Effik International, Brussels, Belgium) is a vaginal mucoadhesive product designed to form a film; that is, a mechanical barrier against colonization by specific pathogens. It can therefore contribute to preventing and managing bacterial vaginosis, notably the prevention of relapses. In such cases, it is used after specific pharmacologic treatment 3 times in the first week. Its administration is to be repeated for 2 consecutive menstrual cycles, mainly after menstruation, whereas nonmenstruating

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patients should take it 3 times in the first week once a month for 3 consecutive months. The first administration should always be immediately preceded by the specific pharmacologic treatment. It may also be useful in the prevention of vaginal colonization by Group B *Streptococcus* (GBS), also called *Streptococcus agalactiae,* which affects 5% to 35% of pregnant women and is associated with a 50% risk of transmission to newborns.[9–13] Newborns infected with GBS can potentially develop fatal conditions, such as pneumonia and sepsis and, more rarely, meningitis.[14–17] GBS may also be responsible for perinatal infections in mothers, such as endometritis.[18]

Three Polybactum formulations have been developed (2 different ovules and a hydro-gel) using 2 main components: polycarbophil, a polymer with excellent bioadhesive properties[19] widely used in products for buccal, ophthalmic, nasal, vaginal, and rectal applications, and lauryl glucoside (LG), a nonionic surfactant.[20] The tensioactive properties of LG may reinforce the barrier effect of the formulation by reducing surface tension, thereby contributing to the mechanical detachment of vaginal pathogens and inhibiting colonization. In addition, the gel contains carbopol for its gel-forming properties, and other inert constituents, whereas the ovules contain Witepsol W35 (Witepsol, Cremer Oleo GmbH & CO. KG, Hamburg, Germany), a hard fat vehicle used to make ovules.

LG belongs to the alkyl (poly)glycoside molecule family consisting of a hydrophobic alkyl residue derived from a fatty alcohol and a hydrophilic saccharide structure derived from dextrose, which are linked through a glycoside bond. Specifically, the alkyl substituents range from 2 to 22 carbons in length, and the D-glycopiranosides consist of glucose-type mono-, di-, tri-, oligo-, or polysaccharides. Regardless of the degree of polymerization, these ingredients are simply called glucosides.[21]

Lauryl (poly)glucoside, International Nomenclature of Cosmetic Ingredients name (European Union Inventory of Cosmetic Ingredients (EU) and Cosmetic Toiletry and Fragrance Association (CTFA)) lauryl glucoside, is listed in the Cosmetic Ingredient Database of the European Union and in the US Food and Drug Administration Voluntary Cosmetic Registration Program. LG, as part of the family of alkyl (poly)glycosides C6-C16, was established to be amongst the compounds generally regarded as safe (notification no. 000237) in 2007. Alkyl glycosides are not included in Annex 1 of the list of dangerous substances of Council Directive 67/548/EEC.

Alkyl (poly)glucosides with chains of various lengths have been investigated in depth to exclude any potential health risks[22–24] within occupational and personal care use. Although these studies are available, the very first issue to be addressed in the development of a novel product for vaginal administration is biocompatibility and irritation potential with regard to the site of use/application. For this reason, a series of in vivo and in vitro studies were conducted to rule out topical hypersensitivity /cytotoxicity of Polybactum, to assess its influence on vaginal flora, to establish its residence time in the vaginal cavity, as well as to quantify any transepithelial absorption that could be associated with systemic toxicity. In this article, we report the findings of the safety profile studies conducted on 2 Polybactum ovule formulations and 1 gel formulation, focusing on intravaginal use.

Materials and Methods

All biocompatibility studies were carried out according to International Organization for Standardization in Pharmaceuticals (ISO) guideline Nos. 10993-5: 2009, 10993-10: 2002, and 10993-12: 2007. The ISO 10993 set entails a series of standards for evaluating the biocompatibility of medical devices.

According to rule 5 of the Annex IX of Directive 93/42/EEC, as amended by Directive 2007/47/EEC, Polybactum is an invasive medical device because it is introduced into a natural orifice of the human body (ie, the vagina). Moreover, it is intended to stay on the vaginal mucosa for more than 60 minutes, but fewer than 30 days. For this reason, it is classified as a IIa medical device.

Annex A of ISO 10993-1 establishes that the biological evaluation of a medical device with these characteristics requires testing of cytotoxicity (ISO 10993-5), irritation (vaginal, ISO 10993-10), and sensitization (ISO 10993-10).

Three formulations of Polybactum were tested:

- Type 1 ovule: Polycarbophil 1.50% w/w, LG 0.04% w/w, and C12-18 acid triglyceride 98.46%;
- Type 2 ovule: Polycarbophil 1.50% w/w, LG 0.1% w/w, hydrogenated coco-glycerides 87.4%, glycerol 5.0%, guar gum 5.0%, and silica 1.0%; and
- Gel: Polycarbophil 1.50% w/w, carbomer 0.5% w/w, LG 0.04% w/w, and glycerin 15%, buffered at pH 3.5 to 4.5.

Effects on vaginal flora

Polybactum ovules and gel (25% w/v and 50% w/v ratio, respectively) were put in direct contact with bacterial suspensions ($>1 \times 10^5$ CFU/mL) for 48 hours. The bacterial suspensions tested were: *Lactobacillus crispatus*, *Lactobacillus jensenii*, *Lactobacillus gasseri*, *Streptococcus agalactiae*, *Neisseria gonorrhoeae,* and *Gardnerella vaginalis.* Additional tests were conducted to determine the MIC of Polybactum Type 1 and 2 ovules and gel. The product was diluted (10%, 25%, or 50% w/v) and put in contact with microbiological suspensions ($>1 \times 10^5$ CFU/mL) of *S agalactiae, L crispatus,* and *G vaginalis* for 24 hours, 48 hours, and 72 hours.

pH-dependent efficacy

We prepared 5 aqueous solutions of melted ovules at different pHs: pH 3, pH 5, pH 7, pH 9, and pH 11. One melted Type 1 or Type 2 ovule was added to 50 mL solution and the induced change in pH was measured by SevenMulti (Mettler Toledo (Mettler Toledo, NextPharma, Limay, France)) pH meter equipped with an InLab Routine Pro (Mettler Toledo, NextPharma, Limay, France) electrode.

Cytotoxicity assay

Cytotoxicity was assessed in Balb 3T3 murine fibroblast cell line. Briefly, cells were exposed to Type 1 and 2 ovules of Polybactum in Dulbecco's modification of Eagle's medium, according to the ISO 10993-5 guideline. After 24 hours, cells were observed under an inverted microscope and cytotoxicity was evaluated by neutral red uptake.

Cell degeneration and malformations were evaluated after 24 hours' incubation on a scale ranging from 0 = no degenerated or malformed cells to 4 = area of degenerated/malformed cells exceeding the specimen more than 1.0 cm). The acceptability criteria for the qualitative evaluation were as follows: negative control ≤ 1 and positive control ≥ 3. The negative control was filter paper placed in the middle of each well, whereas the positive control was 30 mm^2 latex placed in the middle of each well. For the quantitative evaluation, the standard deviation of each group had to be $<18\%$ and positive control percentage cellular vitality had to be $<70\%$. Cytotoxicity was defined as the achievement of a numerical grade >2 and cellular vitality reduction by $>30\%$.

Vaginal irritation

The tests were performed in compliance with ISO 10993-10. This test was performed in 6 female white New Zealand rabbits (3

treated and 3 controls) under the standardized conditions set in the ISO guidelines. All animal experiments were performed in accordance with animal study regulations, and with the approval of the Animal Care and Use Committee. Both Type 1 ovules and Type 2 ovules were tested. The test item (1 ovule) was handled to obtain a bolus of 0.5 g and was introduced with a soft catheter into the vagina of each animal for 5 consecutive days. Control animals were treated with a saline solution (0.9% sodium chloride) injection only under the same conditions. Twenty-four hours after the initial application and immediately before each test, the vaginal opening and perineum of each animal were inspected for signs of discharge, erythema, and edema, and results were recorded, grading the outcome from 0 to 4.

Twenty-four hours after the last application, rabbits were killed and subjected to a macroscopic and microscopic examination. Histologic evaluation was performed on samples collected from 3 areas of each vagina in proximal-distal direction. The vaginal tissue of each animal was graded 0 to 4 for each of the following items: epithelium (0 = normal up to 4 = generalized erosion); leukocyte infiltration (for high power field 0 = absent 4 = more than 100); vascular congestion (0 = absent up to 4 = marked, with disruption of vessels); and edema (0 = absent up to 4 = marked). Hence, the total score ranged from 0 to 16. The total scores were added and divided by the number of animals to obtain an average irritation score. The same calculation was performed for the control group. A total score >9 for the microscopic evaluation in the control tissue could suggest an underlying pathology or, in a control animal, could suggest trauma at dosing. Either situation could require a retest if the other test or control animals exhibited equivalent high scores. The control group average was subtracted from the test group average to obtain the vaginal irritation index.

Sensitization potential

The test was performed in 15 + 3 female albino Guinea pigs according to ISO 10993-10. Both Type 1 ovules and Type 2 ovules were tested. To select the sample dilution, 3 occlusive patches with 0.5 mL undiluted and diluted samples (75% and 50% in sodium chloride) were applied to the back of 3 animals and the dressing was left in place for 24 hours. After bandage removal, no erythema was observed at any of the treated sites.

The experimental design included 1 group of 10 treated animals (Group 1) and one group of 5 control animals (Group 2). The test consisted of an induction phase and a challenge phase according to the relevant guideline. Compounds were classified as positive when at challenge 30% of animals showed a positive reaction (erythema).

Residence time in the vaginal cavity

Residence time was established in young, healthy, female Sprague-Dawley rats weighing 220 to 250 g. A single dose of the test formulation (LG 0.04% or LG 0.1% ovule melted by heating up to body temperature; that is, 37°C, or gel) was administered intravaginally using a cannula in a total volume of 150 μL (volume in excess to ensure even coating of the entire vaginal mucosal surface area). A total of 150 rats were tested, 50 for each formulation. Five animals were assigned to each time point for vaginal sampling, so that each rat underwent only 1 vaginal lavage: +1 hour, +2 hours, +4 hours, +6 hours, +8 hours, +10 hours, +12 hours, +24 hours, +48 hours, and +72 hours. The samples obtained from each lavage were placed on a glass slide and immediately fixed and stained with Alcian blue. Each smear was used to determine the percentage of cells showing polymer gel adhering to their surface as well as the absolute number of cells in each sample, and to perform a semiquantitative analysis of the intensity (density) of global stain expressed as a score between 0 and 10.

Film-forming properties and interaction with vaginal epithelium barrier function

A study using a commercially available model of reconstituted human vaginal epithelium (SkinEthic (SkinEthic, Lyon, France) reconstituted human vaginal epithelium [RHV]) was conducted to assess the film properties and vaginal barrier function of Type 1 and Type 2 ovules. Formulations devoid of LG were used as placebo control (1 for each formulation), saline solution as a negative control, and sodium dodecyl sulfate 1% (SDS 1%) (Sigma-Aldrich, St Louis, Missouri) as a positive control.

The model reproduces vaginal epithelium morphology; it is made of an epithelium formed after 5 days of air-lift culture of an immortalized cell line (A431) in a chemically defined medium. The tissue and media were manufactured in compliance with ISO 9001. Experiments were performed following the supplier's instructions.

Influence on barrier function and fence properties measured by transepithelial electrical resistance

The influence on barrier function and fence properties by transepithelial electrical resistance (TEER) was measured after 6 hours and 24 hours of treatment with Type 1 ovules and before and after 24 to 48 hours of treatment with Type 2 ovules. Measurement time was set up based on the characteristics of the formulation, with the aim of differentiating the 2 compositions. Thirty milligrams of each formulation was applied topically on RHV premoisturized with 30 μL saline solution 1 hour before application, to enable better contact with the epithelial surface. Thirty microliters of positive control (SDS 1%) and negative control were applied in the same conditions. TEER was measured directly on RHV surface before and at the end of treatment, after removal of each product using Millicell-ERS (range, 0–20 kΩ). Measurements were repeated 3 times for each tissue. The t = 0 measurement was set as the baseline and reference value for each single tissue. The blank value (insert without tissue) was subtracted from the sample value (mean of 3 measurements). This result was then corrected considering the tissue surface (0.5 cm^2):

$$\wedge(\textit{mean 3 measurements})\ \text{sample} - \wedge\text{blank} = \wedge \times \text{tissue surface } (0.5\ cm^2).$$

Barrier permeability measured by Lucifer yellow paracellular passage

An amount of 0.5 mL Lucifer yellow (LY) 500 M in saline was introduced into the apical compartment of the insert after exposure to the substance to be tested for the same periods as those listed for TEER. Then, 1 mL saline solution was added to the basolateral compartment. The transport of LY was assessed as a switch from apical to basolateral compartment after an incubation period of 30 minutes at 37°C. Values were read by using a spectrofluorimeter (Tecan Infinite M200 (Tecan Infinite, Vitroscreen, Milan, Italy)) with 428 nm excitation and 535 nm emission. Fluorescence was measured at apical and basolateral level and flux was calculated with the following formula:

$$\text{LY Flux}\,\% = (\text{RFU BL}/\text{RFU AP t} = 0) \times 100,$$

where RFU = fluorescence, bl = basolateral, and ap = apical.

Histomorphologic analysis

Histomorphologic analysis was performed after hematoxylin and eosin (H&E) staining at the end of treatment. Tissue samples were rinsed with saline solution and fixed in buffered 10% formalin. Samples were included in paraffin blocks and sections of 5 μm were obtained. Slides were stained with H&E and evaluated under light microscopy (20×).

Transepithelial passage

The amount of active LG in the vaginal Type 1 and Type 2 ovule prototypes was monitored to assess penetration under exposure

conditions. The 2 vaginal ovule formulations listed above, positive control caffeine 1% (Sigma-Aldrich), and Type 1 ovule placebo were applied topically onto the epithelium surface. The same procedure used for the film-forming properties assessment was followed for the application of 500 (5) mg vaginal ovules over a 24-hour period. The selected receptor fluid was bidistilled water 1 mL/well. A quantity of 1000 μL receptor fluid was collected in plastic tubes at 6 hours and 24 hours and stored at 4°C before being delivered to the analytical laboratories (BioSphere S (Biosphere S, Perugia, Italy) and AR2i (AR2i, Le Plessis Robinson, France)) for LG level determination by mean of a validated gas chromatography–mass spectrometry assay. The assay showed that all validation parameters were satisfactory according to their generally accepted specifications for the concentration range between 0.0004 and 0.01 mg/mL according to International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use standards.[24] The total amount of active LG that penetrated through the epithelium in 24 hours was the sum of the receptor fluids collected at 6 hours and 24 hours.

Statistical analysis

All in vitro experiments were repeated twice, with representative results shown. Statistical analysis was calculated by using Microsoft Office Excel (Redmond, Washington). An unpaired Student *t* test was used. Differences were considered significant at $P \leq 0.05$.

Results and Discussion

Effects on vaginal flora and pH

The first tests were designed to ensure that Polybactum formulations do not affect the normal vaginal flora and pH, which are important to prevent colonization of the vagina by pathogens, such as *G vaginalis and S agalactiae*. Colonization prevention occurs through a number of mechanisms, above all by competition with other microorganisms for nutrients and for adherence to the vaginal epithelium. Additional mechanisms are stimulation of the immune system; reduction in vaginal pH via the production of organic acids, mainly lactic acid; and production of antimicrobial substances, such as bacteriocins and hydrogen peroxide.[3,26]

The vagina is colonized by a number of microorganisms that interact with each other, forming an ecologic niche. The bacterial population varies according to age, menstrual status, pregnancy, sexual activity, medication, and contraceptive use, as well as hygiene.[20,27]

Although the growth of pathogens was affected by Polybactum, the growth of *Lactobacillus* strains was not (all formed $> 10^6$ CFU/mL) (Table I). *G vaginalis* was reduced to no more than 10^2 CFU/mL (Table I) and *S agalactiae* to no more than 100 CFU/mL, whereas *N gonorrhoeae* was reduced to a lesser degree (7-1-8.3 10^4 CFU/mL) (Table II). The test used was able to detect only bacteriostatic and pseudobactericidal effects, so the results cannot be interpreted as a documentation of bactericidal effects Table III and IV.

The MICs determined for Type 1 and Type 2 ovules of Polybactum were as follows: both *G vaginalis* and *S agalactiae* < 10% and *L crispatus* 50% (Table I). The MICs determined for the gel were higher: *G vaginalis* and *S agalactiae* 25%, and *L crispatus* > 50% (Table II). Thereafter, the effect of the gel was not pursued any further.

The next test was designed to verify that Polybactum does not interfere with the maintenance of an acidic environment. The addition of a single Type 1 or Type 2 ovule of Polybactum to solutions maintained at different pHs showed that Polybactum did not increase pH, if anything it actually reduced it slightly. Consequently, the natural acidifying effect of lactobacilli should not be affected by Polybactum (Figure 1).

Biocompatibility

Alkyl (poly)glycosides with C8-C16 alkyl chains belong to the group of very mild surfactants for body cleansing formulations. In a detailed study, the compatibility of alkyl (poly)glycosides was described as a function of pure C chain. In the modified During chamber test, C12 alkyl polyglucoside was the most irritant compound, albeit within the range of mild irritation effects; C8, C10, C14, and C16 alkyl polyglucosides produced lower irritation scores. This is consistent with the findings related to other classes of surfactants.[27]

Table I
Inhibiting activity of Type 1 and Type 2 lauryl glucoside ovules.

Strains	Initial inoculation (CFU/mL)	Contact time (h)	Positive control	Number of surviving cells (CFU/mL)						Minimal inhibitory concentration (% w/v)	
				Type 2 formulation			Type 1 formulation			Type 2 formulation	Type 1 formulation
				10% w/v	25% w/v	50% w/v	10% w/v	25% w/v	50% w/v		
Candida albicans (ATCC 10231)	1.6×105	24	1.6×10^7	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	> 50	> 50
		48	1.6×10^7	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	> 50	> 50
		72	1.6×10^7	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	> 50	> 50
Streptococcus agalactiae (CIP 107.227)	2.6×10^5	24	2.4×10^7	< 100	< 100	< 100	< 100	< 100	< 100	≤ 10	≤ 10
		48	1.5×10^7	< 100	< 100	< 100	< 100	< 100	< 100	≤ 10	≤ 10
		72	5.5×10^7	< 100	< 100	< 100	< 100	< 100	< 100	≤ 10	≤ 10
Neisseria gonorrhoeae (CIP 79.18)	2.0×10^5 (1.2×105)	24	2.9×10^8	$> 3 \times 10^6$	1.3×10^4	2.4×10^3	1.3×10^6 (1.1×10)	6.2×10^5 (3.8×10^5)	2.6×10^4 (2.0×10^5)	25	50 close to 25
		48	1.4×10^8	$> 3 \times 10^6$	3.8×10^5	8.1×10^3	3.7×10^5 ($> 3 \times 10^6$)	6.4×10^5 (3.5×10^5)	< 100 (9×10^4)	50 close to 25	50 close to 25
		72	$> 3 \times 10^8$	$> 3 \times 10^6$	5.5×10^5	1.5×10^4	6.9×10^5 ($> 3 \times 10^6$)	6.2×10^5 (5.6×10^5)	2.8×10^5 (5.0×10^4)	50 close to 25 %	50 close to 25
Lactobacillus crispatus (CIP 103.603)	1.6×10^5	24	$> 3 \times 10^8$	$> 3 \times 10^6$	1.2×10^6	5×10^4	$> 3 \times 10^6$	1.1×10^6	5×10^5	50	> 50 close to 50
		48	$> 3 \times 10^8$	$> 3 \times 10^6$	1.5×10^6	1.1×10^5	1×10^5	$> 3 \times 10^6$	5×10^4	50	50
		72	$> 3 \times 10^8$	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	2.6×10^6	9.4×10^5	1.4×10^4	> 50	50
Gardnerella vaginalis (CIP 70.74)	2.1×10^5	24	1.1×10^6	4×10^2	< 100	< 100	< 100	< 100	< 100	≤ 10	≤ 10
		48	1.6×10^7	2.1×10^4	< 100	< 100	< 100	< 100	< 100	≤ 10	≤ 10
		72	2.9×10^7	< 100	< 100	< 100	< 100	< 100	< 100	≤ 10	≤ 10

Table II
Inhibiting activity of lauryl glucoside gel.

Strain	Initial inoculation (CFU/mL)	Contact time (h)	Positive control	No. of surviving cells (CFU/mL)			MIC (% w/v)
				10% (w/v)	25% (w/v)	50% (w/v)	
Candida albicans (ATCC 10231)	2.6×10^5	24	0.9×10^7	1.9×10^6	1.0×10^6	1.9×10^5	50
		48	1.2×10^7	$> 3 \times 10^6$	1.8×10^6	1.1×10^6	> 50
		72	1.8×10^7	$> 3 \times 10^6$	$> 3 \times 10^6$	2.0×10^6	> 50
Streptococcus agalactiae (CIP 107.227)	0.7×10^5	24	3.8×10^7	$7.2\ 10^5$	< 100	< 100	25 close to 10
		48	2.5×10^7	$> 3 \times 10^6$	< 100	< 100	25
		72	4.6×10^7	$> 3 \times 10^6$	< 100	< 100	25
Neisseria gonorrhoeae (CIP 79.18)	1.2×10^5	24	1.1×10^6	$> 3 \times 10^6$	4.4×10^5	< 100	50 close to 25
		48	8.9×10^7	$> 3 \times 10^6$	4.5×10^5	< 100	50 close to 25
		72	1.3×10^6	$> 3 \times 10^6$	8.0×10^5	< 100	50
Lactobacillus crispatus (CIP 103.603)	1.2×10^5	24	1.1×10^8	2.4×10^6	1.9×10^6	1.3×10^6	> 50
		48	2.6×10^8	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	> 50
		72	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	$> 3 \times 10^6$	> 50
Gardnerella vaginalis (CIP 70.74)	1.1×10^5	24	1.3×10^6	1.0×10^5	< 100	< 100	≤ 10
		48	1.7×10^6	1.4×10^5	< 100	< 100	≈ 10
		72	1.7×10^6	2.3×10^5	< 100	< 100	25 close to 10

On the other hand, the in vitro test on the choreonallantoic membrane of fertilized hens' eggs as an alternative to Draize's mucous membrane compatibility test, shows a monotonic decrease from C8 to C14 alkyl polyglucosides within the range of mild irritation effects. The commercial alkyl polyglycoside products (Plantacare 1200, Plantacare 2000, and Plantacare 818 (Plantacare, BASF, Levallois, France)) with mixed carbon chain length have the best overall compatibility with the relatively high proportion of long-chain alkyl polyglucosides.[27]

Regarding clinical studies, the sensitization potential of 0.5%, 0.75%, and 1.8% active ingredient decyl glucoside (in formulation), 5% active ingredient aqueous decyl and LG, and 1% active ingredient aqueous coco-glucoside was evaluated in a human repeated insult patch test.[21] The ingredients were not irritating or sensitizing.

Because LG is not considered to be a skin irritant or a mucous membrane irritant and sensitizer, and being included in the Polybactum formulation (0.04%) at a very low concentration, we did not expect any specific issue from a biocompatibility point of view.

The next step was to confirm biocompatibility by verifying the absence of cytotoxic, irritant, and sensitizing effects of Polybactum in vitro and in vivo. The exposure of fibroblasts to Type 1 ovules of Polybactum disclosed a negligible reduction in vitality, accounting for no more than 2.37%; the exposure to Type 2 ovules disclosed a slightly higher reduction in vitality, which was still small enough to determine that the formulation was not cytotoxic (–9.22%).

Regarding vaginal irritation, the results are detailed in Figure 2. There were no macroscopic findings. According to microscopic findings (not shown), the mean irritation capacity of Type 1 ovules was 3.67 versus 2.67 related to sodium chloride, whereas the mean irritation capacity of Type 2 ovules was similar to that of sodium chloride. Whereas Type 1 ovules were reported to be minimally irritating, Type 2 ovules were found not to be irritating at all. The lack of dose response with regard to LG suggested that minimal irritation with Type 1 ovules may have been a chance finding, rather than the expression of an irritation potential that would increase with additional use.

Moreover, none of the animals exposed to the Type 1 or Type 2 ovules of Polybactum exhibited signs of sensitization. Thus, we concluded that Polybactum ovules were not cytotoxic or irritating, nor did they induce sensitization, thus confirming the original hypothesis.

Residence time in the vaginal cavity

Residence time of the 0.04% ovule in the vaginal cavity was very satisfactory: the mean (SD) percentage of cells that still had polymer adhering to them at 48 hours was 99% (2.1%) and at 72 hours 91% (8.1%); mean (SD) staining was still intense, at 7.4 (1.5) and 7.4 (1.3), respectively (Figure 3). Residence time of the 0.1% ovule in the vaginal cavity was similar: The mean (SD) percentage of cells that still had polymer adhering to them at 48 hours was 95% (6%) and at 72 hours 93.4% (9.8%). Mean (SD) staining was not quite so intense, at 6.4 (1.1) and 7.2 (1.1), respectively.

Table III
MICs found for vaginal ovules.

Strain	Contact time (h)	MIC (% w/v)	
		Type 2 ovule	Type 1 ovule
Candida albicans	24	> 50	> 50
	48	> 50	> 50
	72	> 50	> 50
Streptococcus agalactiae	24	≤ 10	≤ 10
	48	≤ 10	≤ 10
	72	10	≤ 10
Neisseria gonorrhoeae	24	25	50 close to 25
	48	50 close to 25	50 close to 25
	72	50 close to 25	50 close to 25
Lactobacillus crispatus	24	50	> 50 close to 50
	48	50	50
	72	> 50	50
Gardnerella vaginalis	24	≤ 10	≤ 10
	48	≤ 10	≤ 10
	72	≤ 10	≤ 10

Table IV
MICs found for vaginal gel.

Strain	Contact time (h)	MIC (% w/v)
Candida albicans	24	50
	48	> 50
	72	> 50
Streptococcus agalactiae	24	25 close to 10
	48	25
	72	25
Neisseria gonorrhoeae	24	50 close to 25
	48	50 close to 25
	72	50
Lactobacillus crispatus	24	> 50
	48	> 50
	72	> 50
Gardnerella vaginalis	24	≤ 10
	48	≈ 10
	72	25 close to 10

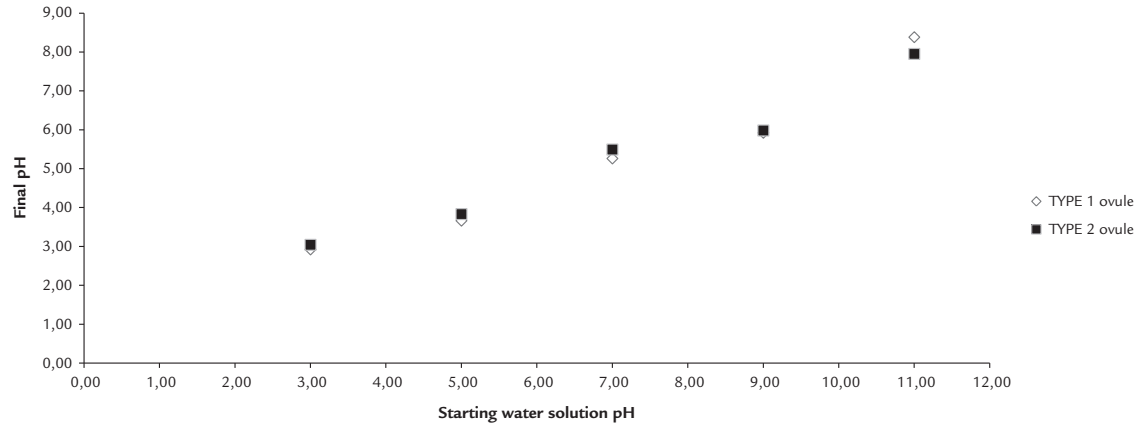


Fig. 1. Changes in pH induced by the introduction of a single Type 1 or Type 2 Polybactum (Effik International, Brussels, Belgium) ovule into 50 mL solutions at different pHs.

The residence time of the gel was not quite as good as the residence time of the ovules: The mean (SD) percentage of cells that still had polymer adhering to them at 48 hours was 87.2% (18.1%) and at 72 hours 88.2% (5.0%). However, mean (SD) staining was still intense, at 7.4 (2.4) and 7.8 (1.4), respectively.

In vitro effects on vaginal barrier and film-forming properties

Caprylyl glucoside increases the absorption of poorly absorbed drugs (eg, insulin) both in vitro across human carcinoma monolayers and in vivo through mucosal membranes. The effect of other alkyl glycosides, including decyl glucoside and LG, on mucosal penetration was also evaluated. A 5% solution of decyl glucoside also enhanced the buccal absorption of insulin, whereas 5% LG did not have the same effect. Researchers have stated that there is no consistent relationship between alkyl chain length and penetration enhancement.[28]

To confirm that LG does not affect vaginal mucosa barrier and fence properties, TEER measurements were used. TEER is the measure of the movement of ions across the paracellular pathway regulated by polarized plasma membrane surfaces and by cell-to-cell tight junctions, which, together, prevent movement of solutes and ions across the epithelium. TEER is an indirect assessment of tight junction stability and, consequently, is a direct measure of barrier function in epithelial tissue because it reflects the global resistance of the barrier linked both to the structure and to epithelial thickness. Maintenance of stability and electrical resistance of an epithelium is critical for essential physiological processes. Therefore, significant changes in TEER may be an early expression of cell damage and can be considered as a complementary parameter.

TEER was directly measured on RHV surfaces before and at the end of treatment after product removal.

Figure 4 shows that none of the Polybactum formulations influenced epithelial fence properties. Moreover, their effects were considerably lower than the effects of the positive control, which ranged between –58% and –67%. The effects of Type 2 Polybactum ovules were considerably lower, ranging between –16% and –44% and Type 1 Polybactum ovules actually increased resistance by 78% after 24 hours ($P < 0.01$ vs placebo), thus exhibiting good film-forming properties.

Due to the superiority of the film-forming properties exhibited by Type 1 ovules, further studies on the effects on vaginal epithelium were performed only with this formulation.

Barrier integrity was assessed in-depth by means of the LY assay. LY is a fluorescent dye impermeable to the cell membrane, which is used to study the paracellular permeability of a substance. When the junctions are unbroken,

LY has a very low permeability; if the joints are damaged, LY flow will be much higher. Therefore, this assay is used to verify the integrity of cell junctions in the presence of the substance to be evaluated. The results obtained with this dye showed that only the SDS 1% increased permeability, increasing LY assay 2-fold (Figure 5).

The ultimate confirmation that Polybactum did not have any untoward effects on vaginal epithelium was provided by the histomorphologic evaluation based on H&E staining. Only SDS 1% produced loss in cell-to-cell connections and structural modifications in the whole tissue at 6 hours. Only slight changes were

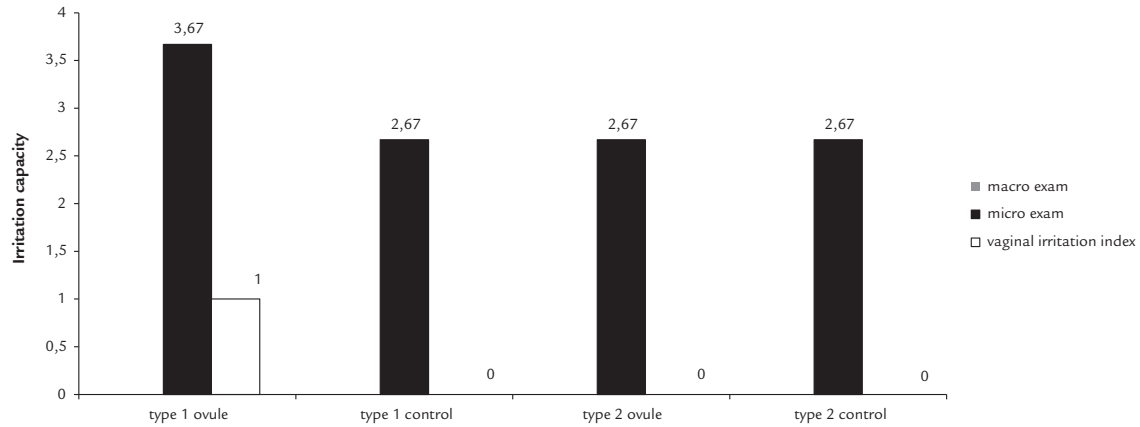


Fig. 2. Severity of macroscopic and microscopic findings, and vaginal irritation index expressed on a 5-item semiquantitative scale after exposure to 0.5 g Type 1 or Type 2 Polybactum (Effik International, Brussels, Belgium) ovules introduced daily for 5 days into the vagina of 3 white New Zealand rabbits and to sodium chloride injection in 3 control animals.

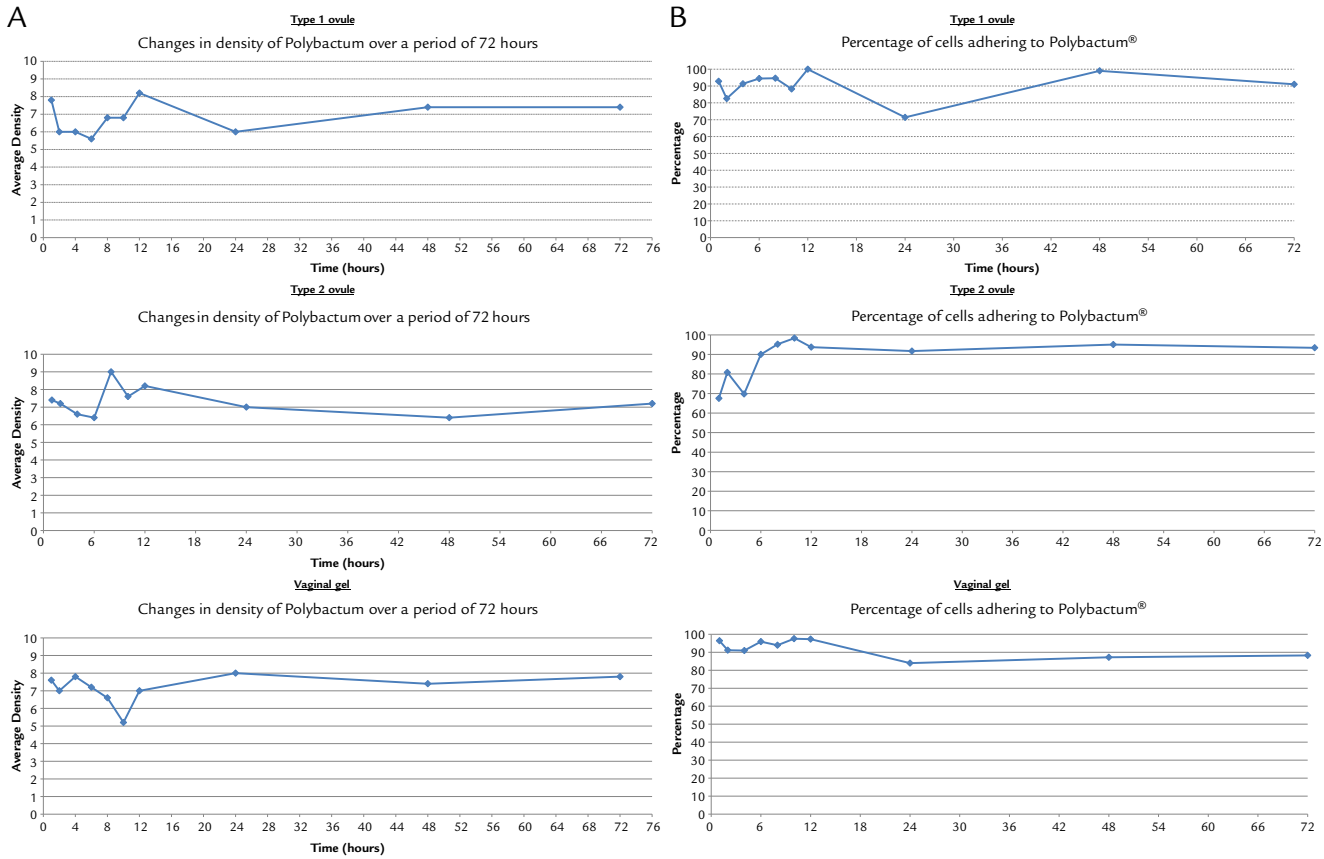


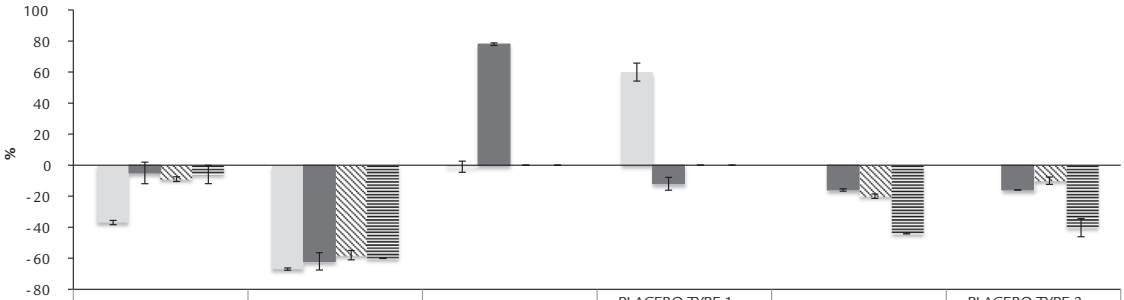
Fig. 3. (A) Mean (SD) staining intensity score as an index of ovule density over 72 hours after intravaginal administration. (B) Mean (SD) percentage of cells showing polymer adhering to their surface over 72 hours after intravaginal administration.

observed with placebo and Type 1 ovules. At 24 hours, changes progressed to intermediate modifications with reduced connection among cells both with placebo and Type 1 ovules (Figure 6).

Transepithelial passage

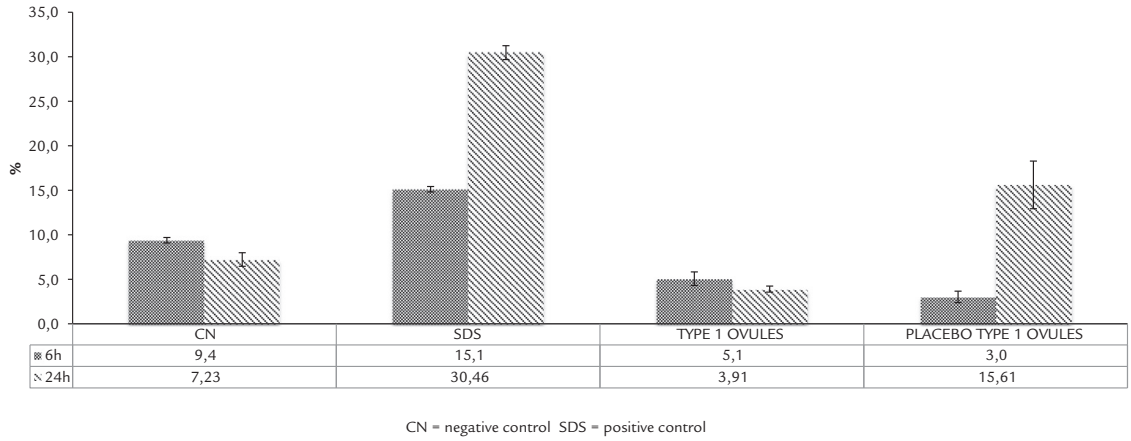
The dermal penetration of caprylyl/capryl glucoside (dilution at 10% in Hanks' buffered salt solution [pH 6.5]) was evaluated in vitro by using human skin. The mean absorbed dose of caprylyl/capryl glucoside, as the sum of the amounts found in viable epidermis, dermis, and receptor medium, was 0.01%.[29] Literature studies show that glucoside hydrolases occur in human skin, stomach, intestine, and mucosa.[30]

The last issue addressed was the potential for systemic toxicity due to transvaginal passage of LG, although none was expected. Type 1 and Type 2 ovules were applied topically onto the epithelium surface and LG levels were determined in the basolateral compartment after 6 hours and 24 hours, using absorption of caffeine as a positive control. To avoid analytical interference and according to previous testing with LG, the selected receptor fluid was bidistilled water 1 mL/well. Type 1 ovule placebo, without LG, was tested in parallel to exclude any analytical interferences of the raw materials with the detection of LG. Negligible amounts of LG were detected after Type 1 and Type 2 ovule application, whereas large amounts of caffeine were absorbed (68.9%) (Table V). Thus, we conclude that LG was not



	CN	SDS 1%	TYPE 1 OVULES	PLACEBO TYPE 1 OVULES	TYPE 2 OVULES	PLACEBO TYPE 2 OVULES
6h	-37	-67	-1	60	0	0
24h	-5	-62	78	-12	-16	-16
48h	-9	-58	0	0	-20	-10
72h	-6	-60	0	0	-44	-40

Fig. 4. Changes in transepithelial electrical resistance (%) as a measure of influence on barrier function and fence properties of vaginal epithelium, in the 72 hours following application of negative control (CN), positive control (SDS 1%), Type 1 and Type 2 ovules, and relative placebos.



	CN	SDS	TYPE 1 OVULES	PLACEBO TYPE 1 OVULES
6h	9,4	15,1	5,1	3,0
24h	7,23	30,46	3,91	15,61

CN = negative control SDS = positive control

Fig. 5. Changes in the extent of Lucifer yellow flux (%) as a measure of permeability 6 and 24 hours after exposure to Type 1 Polybactum (Effik International, Brussels, Belgium) ovule and negative control (CN) and positive control (SDS) formulations.

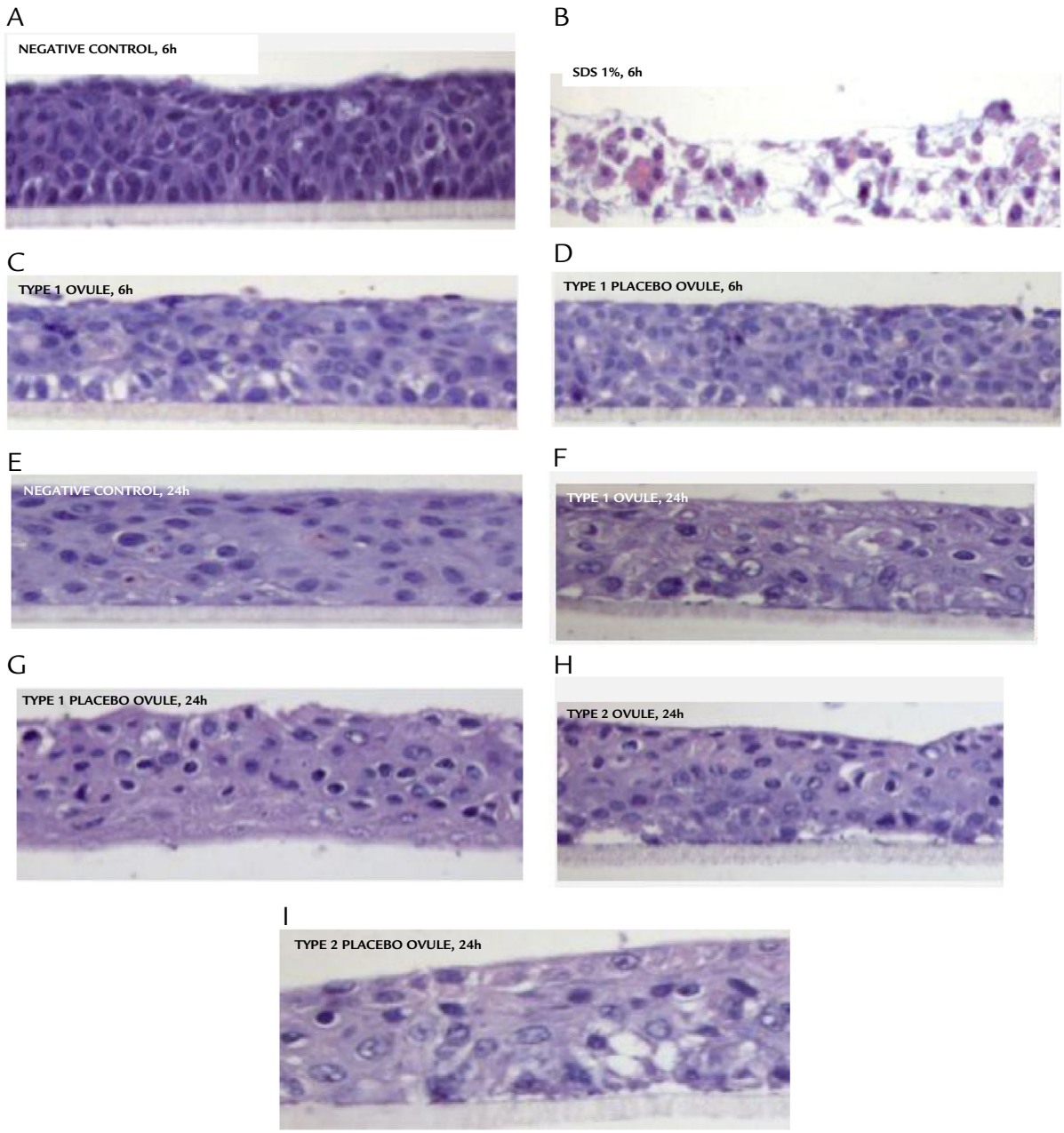


Fig. 6. Histomorphologic analysis by haematoxilin and eosin staining after product treatments to confirm the results obtained and for a deeper understanding of the type of interaction between the product and the living tissue.

Table V
Absorption rates through reconstituted human vaginal epithelium (RHV).

Product	Dose applied		Lauryl glucoside % determined after penetration/diffusion on RHV in basolateral compartment		
	Quantity applied (formulation) (mg/0.5 cm^2) Mean (SD)	Quantity applied (Lauryl glucoside) (mg/0.5 cm^2)	6 h	24 h	Total receptor fluid
Type 0.095	2 ovule	501 (3)	0.5	0.040	0.055
Type 0.187	1 ovule	504 (1)	0.2	0.098	0.089
Placebo	496	0.0	0.000	0.000	0.000
Caffeine 1%	502 (2)	5.02*	36.78	32.19	68.97

* Caffeine, not lauryl glucoside.

absorbed through the vaginal epithelium. This excludes the risk of systemic effects.

Conclusions

Polybactum is a vaginal mucoadhesive product designed to form a film that acts as a mechanical barrier against colonization by pathogens. This article reports preclinical studies addressing a number of important safety profile issues. Polybactum was shown in our study to have no cytotoxic, irritant, or sensitizing effects. It also did not impair natural defenses because it did not affect the normal vaginal flora, pH, and/or epithelium fence properties. Type 1 ovule formulation was also found to have film-forming properties. Lastly, it was noted that although residence time was good, with approximately 90% of cells showing adhering polymer 72 hours after administration, permeation of LG (the only component that is not already used in vaginal formulations) through RHV was negligible. This finding rules out the risk for systemic exposure to a substance with potentially unknown untoward effects. The safety profile of the other constituents is well known. This favorable preclinical profile is encouraging and warrants further studies on Polybactum Type 1 ovules as adjunct therapy for important vaginal infections, as well as for the prevention of recurring bacterial vaginosis and *S agalactiae* colonization.

Acknowledgments

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Conflicts of Interest

Effik International provided funding for the studies reported in this article. L.I. Ardolino is employed by Effik Italia, a company belonging to Effik Group headed by Effik International. The authors have indicated that they have no other conflicts of interest regarding the content of this article.

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