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The Composition of Cell Wall Skeleton and Outermost Lipids of *Mycobacterium vaccae* is Modified by Ethambutol Treatment

ANNA RUMIJOWSKA-GALEWICZ[1*], MAŁGORZATA KORYCKA-MACHAŁA[1], KATARZYNA LISOWSKA[2] and JAROSŁAW DZIADEK[1]

[1] Institute for Medical Biology Polish Academy of Sciences, Łódź, Poland
[2] Department of Industrial Microbiology and Biotechnology, University of Łódź

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Abstract

Ethambutol (EMB) is a first line drug in tuberculosis treatment inhibiting the biosynthesis of arabinogalactan, which is a component of the mycobacterial cell wall. The growth of *Mycobacterium vaccae* cells in the presence of EMB increases cell wall permeability, which was monitored by β-sitosterol biotransformation. GC/MS and GLC/MS (gas chromatography/mass spectrometry) analysis revealed dramatic changes in the content of covalently bound mycolic acids and in molar ratio galactose (Gal) to arabinose (Ara) in the cell envelopes of EMB-treated cells. The detected variations in the compositions of fatty acids indicate that both the cell wall skeleton and outer layer (free lipids) are decomposed due to EMB treatment.

Key words: ethambutol, mycobacterial cell wall skeleton, permeability

Introduction

Mycobacteria are surrounded by a cell wall with the unique structural and functional characteristics. Richness in lipidic compounds, as well as their composition and organization, are the most distinctive features of the mycobacterial cell wall (Daffe and Draper, 1998). Three structures: peptidoglycan, arabinogalactan and mycolic acids are covalently associated and form the mycobacterial cell wall skeleton (CWS). Arabinogalactan is composed of 3 arabinan chains (composed of 27 D-arabinofuranosyl units each) attached to the homogalactan core (consisting of the 32 D-galactofuranosyl units) of linear alternating 5- and 6-linked β-D-galactofuranosyl residues. Arabinan chains are composed of linear α-D-arabinofuranosyl residues with branching produced by 3,5-linked α-D-arabinofuranosyl units substituted at both positions by α-D-arabinosyl residues. The nonreducing termini of arabinan consists of a branched hexaarabinofuranosyl motif to which mycolic acids are attached (Crick *et al.*, 2001; McNeil, 1999).

Mycolic acids, the most prominent constituent of the mycobacterial cell wall, occur as a close-packed quasicrystalline layer perpendicular to peptidoglycan (Nikaido *et al.*, 1993). This highly ordered structure of very low fluidity (Liu *et al.*, 1996) is thought to be responsible for drug and solute impenetrability, which is one of the multiple drug resistance factors of pathogenic mycobacteria, it also limits the influx rate of compounds essential for biotechnological processes.

The outer leaflet of the mycobacterial lipid bilayer consists mainly of easily extractable, polar lipids, including a number of species- and type-specific glycolipids, glycerophospholipids, sulpholipids, glycopeptidolipids (GPL), phenolic glycolipids (PGL), lipoarabinomannan (LAM), trehalose dimycolate (TDM), phtiocerol dimycocerosate, and others (Rastogi, 1991; Brennan and Nikaido, 1995; Liu *et al.*, 1995; Daffe and Draper, 1998). These lipids form a unique asymmetric bilayer, which is considered to be responsible for the extremely low permeability of the cell wall to hydrophilic and hydrophobic substances (Jarlier and Nikaido, 1990; Liu *et al.*, 1995; Nikaido *et al.*, 1993). Hydrophobic compounds should cross the mycobacterial cell wall easily, as it is assumed that they dissolve in the lipid domain and redissolve in the aqueous phase on the other side of the cell (Nikaido and

* Corresponding author: A. Rumijowska-Galewicz, Institute for Medical Biology Polish Academy of Sciences, 93-232 Łódź, Lodowa 106, Poland; phone: (48) 42 2723625; fax: (48) 42 2723630; **e-mail: arumijowska@cbm.pan.pl**

Jarlier, 1991). However, the data concerning the physical organization of mycobacterial cell wall lipids indicate that outermost lipid leaflet may be the permeability barrier for the hydrophobic solutes, too. Very few papers have been published on the penetration of lipophilic materials (mainly antimycobacterial agents) through the mycobacterial cell wall. Generally, the compounds, which enhance the lipophilicity of substances increase the penetration rate through the lipid domain of the cell wall (Connel and Nikaido, 1994). The permeability of the outer leaflet of the mycobacterial cell wall bilayer may be enhanced by specific inhibitors, interfering with biosynthesis of mycobacterial cell wall components (David *et al.*, 1988; Rumijowska-Galewicz *et al.*, 2000).

Mycobacterium vaccae B 3805 is a model organism for studying the penetration of lipophilic substrates through the lipidic interior and other cell wall barriers. This microorganism can selectively degrade the sterol side-chain, resulting in the accumulation of 4-androsten-3,17-dione (AD) and 1,4-androstadien-3,17-dione (ADD). As the enzyme system involved in this transformation is intracellular, the accumulation of AD(D) depends on the sterol penetration rate through the cell envelope (Korycka-Machała *et al.*, 2005; Lisowska *et al.*, 1996; Sedlaczek *et al.*, 1999).

Ethambutol (EMB) is a synthetic compound that has been known for its antimycobacterial activity recommended for tuberculosis treatment as a first-line drug. Ethambutol effects are pleiotropic and several hypotheses of their action have been proposed. EMB affects primarily the biosynthesis of arabinan in the arabinogalactan and sequentially lipoarabinomannan in the cell wall of *M. tuberculosis* (Deng *et al.*, 1995; Ramalho *et al.*, 2004).

In the present study we have shown that EMB treatment increases the permeability of *M. vaccae* B 3805 cell wall by decomposition of arabinogalactan, mycolic acids and outer layer free lipids.

Experimental

Materials and Methods

Microorganism and medium. The *Mycobacterium vaccae* B 3805 able to selective cleavage of the β-sitosterol side chain yielding androst-4-ene-3,17-dione (AD) with trace amount of androsta-1,4-diene-3,17-dione (ADD) was cultured in NB (nutrient broth (Difco) 8.0 g/l and glucose 10.0 g/l supplemented with 0.2% (v/v) Tween 80) medium supplemented with β-sitosterol or EMB when indicated.

Sterols and steroids. β-sitosterol (Triple Crown, Sweden) was prepared as previously described (Sedlaczek *et al.*, 1994). Cholesterol (Serva) and androst-1-ene-3,11,17-trione (Sigma) were used as internal standards for quantitative determination of steroids by GC (for β-sitosterol and AD respectively). AD (4-androsten-3,17-dione) from Koch-Light was used as a standard.

Growth and β-sitosterol transformation. *M. vaccae* was growing in NB medium at 32°C with shaking. At the time of inoculation, β-sitosterol (0.2 g/l) and the EMB were added to the medium. To determine the cell dry mass, at the beginning of the experiments and at 24-h intervals, samples were withdrawn from the culture. The progress in β-sitosterol side chain degradation was determined in 2-ml culture samples to which cholesterol and androst-1-ene-3,11,17-trione as internal standards were added and extracted with chloroform and analyzed by chromatography as described previously (Rumijowska *et al.*, 1997). The selective side chain degradation of β-sitosterol is catalysed intracellularly by an enzyme complex (Szentirmai, 1990). The substrate must permeate the cell wall to be transformed to androstene derivatives AD(D). Therefore the rate of AD(D) formation and accumulation depends on cell wall permeability.

Isolation, purification and analysis of the cell wall skeleton and arabinogalactan. The cell wall skeleton of control and EMB-treated cells was isolated according to procedures described by Hunter *et al.* (1989) and Azuma *et al.* (1974) and modified by Sedlaczek *et al.* (1999). Purified CWS preparation was subjected to methanolysis according to Minnikin *et al.* (1980) and analysed for mycolic acid methyl esters (MAMEs). Mycolic acids covalently bounded to the cell wall skeleton were removed by the alkali hydrolysis with 0.5% KOH in ethanol at 37°C for 48 hours and centrifuged at 30 000 × g. Supernatant was evaporated and remaining mycolic acids were analysed by weighting and GC/MS. Arabinogalactan sugars were analyzed as their alditol acetates by GLC/MS. A solution of the sample (with standard-Rha) in 2 M trifluoroacetic acid was kept at 120°C for 2 h, then the acid was evaporated. The sugars in the hydrolysate were converted into alditol acetates by overnight treatment with aqueous sodium borohydride. After the addition of acetic acid, boric acid were removed as the methyl ester by codistillation with methanol and then the alditols were acetylated with acetic anhidride/pyridine (1:1) at 100°C for 1 h; the products was analyzed by GLC/MS.

Lipid analysis. (a) Extraction and methanolysis of free lipids. Lyophilized control and inhibitor-treated cells (4 g) were extracted three times with chloroform/methanol (2:1, v/v) for 48 h at room temperature. Extracts containing free lipids were concentrated, washed with water, evaporated to dryness under nitrogen and analyzed. Defatted cells were dried and kept for the further analysis. Free lipids were subjected to methanolysis according to Minnikin *et al.* (1980) and analyzed by GC/MS (Rumijowska *et al.*, 1997). (b) Separa-

tion of lipid fractions. Crude free lipids (250 mg) from control and ethambutol-treated cells were fractionated on a column of activated silica gel (70–230 mesh, Merck); chloroform (800 ml), acetone (1600 ml), and methanol (800 ml) were used as eluents (Gamian *et al.*, 1996). The eluates were monitored by TLC. Fractions containing similar compounds eluted by the same solvent were combined and analyzed. (c) Analysis of mycolic and fatty acids. Mycolic acids were determined in methanolysates of cell wall skeleton (CWS), delipidated cells and free lipids. Fatty acids were analyzed in free lipids, lipid fractions eluted from the silica gel 60 column, and the after-growth medium of control and EMB-treated cultures. The mycolic acid methyl esters (MAMEs) and fatty acid methyl esters (FAMEs) were determined by weighing, and subjected to GC/MS and pyrolysis GC procedures for the analysis of their fatty acids and related compounds. The amount of mycolic acids is defined by measuring of docosanoic acid (22:0). Similar quantities of this compound may result from similar content of mycolic acids in the analyzed preparations. Docosanoic acid is formed as methyl ester *in situ* upon pyrolysis of the complex mycolic acid structure in the heated injector of gas chromatograph (Garcia-Borcello *et al.*, 1993). The α-branch is released, which in *M. vaccae* contains mainly 22 carbon atoms. 2-Eicosanol, a secondary alcohol present in mycobacteria that contain carboxymycolates (Valero-Guillen and Martin Luengo, 1986), was another component derived directly from mycolic acid cleavage.

Results

Ethambutol as a compound increasing the biotransformation activity. Intracellular β-sitosterol biotransformation to AD(D) by *M. vaccae* B 3805 depends on the cell wall permeability for the substrate. We analyzed the accumulation of AD(D) in *M. vaccae* culture to observe the effect of EMB on cell wall permeability. We have previously shown that EMB concentrations ranging from 2–50 μg/ml have only a minimal effect on the cell growth (Korycka-Machała *et al.*, 2005). In this work we found that the most effective inhibitor concentrations ranged from 5 to 15 μg/ml (Fig. 1). The amount of AD(D) detected in *M. vaccae* culture supplemented with 10 μg/ml of EMB increased twofold compared to the control. Since the biotransformation efficiency depends on substrate penetration through the cell envelope of *M. vaccae* we conclude that EMB in concentration of 10 μg/ml increases the permeability of the cell wall for steroid compounds approximately two-fold. It is very likely that the increased permeability of the cell wall is due to alterations in the cell wall architecture and cell wall lipid composition caused by the EMB.

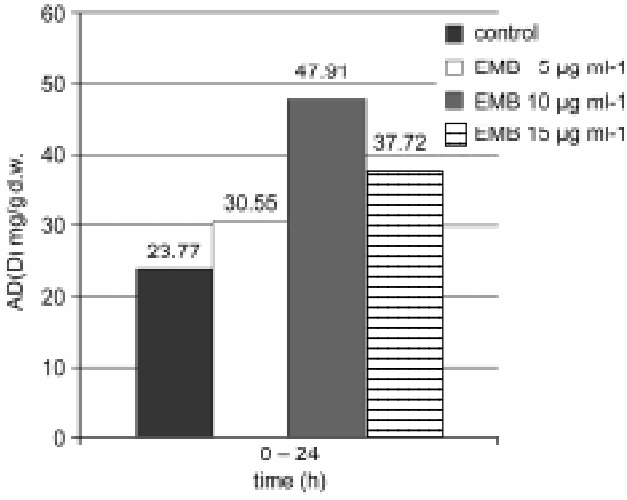

Fig. 1. The activity of *M. vaccae* cells in β-sitosterol transformation in 24 hour of biotransformation process.

The amount of AD formed during this time was divided by the cell biomass. The black bar represents control *M. vaccae* B3805 culture without EMB. The white gray and striped bars represent *M. vaccae* B3805 cultures supplemented with 5, 10 and 15 mg/ml of EMB, respectively.

The effect of ethambutol on cell wall skeleton of *M. vaccae* B 3805. To detect the cell wall alteration connected to increased permeability, arabinogalactan was analyzed as a known ethambutol target. Five grams of the lyophilized control and exposed to ethambutol (10 μg/ml) cells were used to obtain 822 and 537 mg of cell wall skeleton (CWS), respectively (Table I). The lower amount of CWS isolated from bacilli exposed to ethambutol may be related to inhibition of the arabinan biosynthesis which is a component of the arabinogalactan (Takayama and Kilburn, 1989). By using alkali hydrolysis we were able to isolate 10.95 mg and 8.04 mg of the mycolic acids from the same amount of CWS of control and EMB-treated cells, respectively. This means that in the presence of ethambutol, the total amount of mycolic acids bound to arabinogalactan-peptidoglycan complex was about 35% lower compared to the control cells. To investigate the modifications in the arabinogalactan, the mycolic acids were hydrolyzed and the sugars alditols of AG were analyzed. The presence of EMB (10 μg/ml) decreased arabinose content in AG. GLC/MS analysis of alditol acetate derived from arabinogalactan revealed that molar ratio Ara:Gal decreased from 1.48:1

Table I
Changes in the cell wall skeleton under action of ethambutol

	Control cells	Cells exposed to ethambutol
CWS (mg) – obtained from 5 g of lyophilized cells	822.0	537.0
Bound mycolic acids (mg) obtained from 100 mg of CWS	10.95	8.04
Molar ratio Ara:Gal	1.48:1	0.78:1

in the control cells to 0.78:1 in the EMB-treated cells. These modifications resulted in decreasing of the arabinan units substituted by mycoloyl residues. The observed modifications in the cell wall skeleton like decreased molar ratio Ara:Gal and lower amount of the covalently bound mycolic acids are likely responsible for the increased cell wall permeability.

To investigate if EMB affects the outer parts of *M. vaccae* cell wall a chemical analysis of the defatted cells and free lipids was performed.

The effect of ethambutol on fatty acid composition of *M. vaccae* B 3805 defatted cells. It is generally accepted that mycolic acids are an important permeability barrier in the mycobacterial cell wall. We used 4 g of the lyophilized control and EMB-treated cells to isolate 2.94 and 2.47 g of delipidated cells containing mycolic acids covalently bound to arabinogalactan. Defatted cells were subjected to methanolysis and resulted products (docosanoic acid and 2-eicosanol) were identified by GC/MS. The FAMEs analysis revealed the significant changes in the quantity of the separate acids. We observed the decreased amount of C22:0 in the EMB-treated bacilli comparing to control cells (49.8% compared to 59.5%). The lower amount of the mycolic acids covalently bounded to the arabinogalactan in EMB-treated cells agrees with our observations of the cell wall skeleton analysis (see above). Moreover, the culture supernatant of ethambutol treated cells, but not control culture supernatant, revealed increased amount of docosanoic acid (Table II) likely released from *M. vaccae* cells growing in the presence of EMB.

To investigate the detected modification in details the cell envelope analysis of the free lipids was performed.

The effect of ethambutol on fatty acid composition of *M. vaccae* B 3805 cell wall free lipids. The most external barrier for environmental compounds in mycobacterial cells is the outer leaflet asymmetric bilayer. We used chloroform : methanol 2:1 to extract free lipids from control and EMB-treated lyophilized cells. Free lipids containing 16–22 carbons fatty acids removed from cells were identified by GC/MS. The total content of fatty acids in the control was higher than in the EMB-treated cells (2948.9 and 2039.8 nmol/mg, respectively). Chloroform/methanol extracts contain a variety of lipids including neutral lipids, glycolipids and phospholipids. To analyze each of these groups, crude free lipids were fractionated on a silica gel by successive use of chloroform, acetone and methanol as eluting solvents. Chloroform-derived material contains mainly neutral lipids, *i.e.*, glycerides, waxes, hydrocarbons, carotenoids, fatty acids and others, glycolipids and phospholipids were eluted by acetone and methanol, respectively (Koul and Prasad, 1996). The differences between control and EMB-treated cells were detected in acetone and methanol-eluted lipids. This suggests that ethambutol affects mainly glycolipids and phospholipids of the mycobacterial cell wall outer layer. The fatty acids profile of the lipid fractions are shown in Table III. Significant quantitative differences in fatty acid patterns of the analyzed preparations occurred in glycolipids eluted by acetone composed mainly with methyl-branched fatty acids (2 Me 18:1), octadecanoic (18:0) and docosanoic acids (22:0).

Table II
Fatty acid composition of post culture media of control, and ethambutol-containing cultures of *M. vaccae* B 3805

Component	Fatty acids obtained from:			
	control post culture medium		ethambutol-containing post culture medium	
	Nmol/mg	mol %	Nmol/mg	mol %
16:1	53.7 ± 2.1	4.5		
16:0	36.2 ± 1.3	3.0	46.2 ± 1.9	3.3
18:1	42.1 ± 1.7	3.5	410.5 ± 28.6	29.7
2-Me 18:1	403.8 ± 25.3	34.2		
20:0	242.0 ± 12.5	20.5	265.9 ± 11.7	19.3
22:0	404.5 ± 28.9	34.3	658.1 ± 39.2	47.7
Total	1182.3		1380.7	100

Table III
Fatty acid composition of free lipid fractions from control and ethambutol-treated cells of *M. vaccae* B 3805

Component	Control cells		Cells exposed to ethambutol	
	Nmol/mg	mol %	Nmol/mg	mol %
(a) Chloroform-eluted lipids				
16:0	128.3 ± 3.6	34.6	99.8 ± 3.2	31.7
18:1	150.7 ± 7.2	40.7	132.8 ± 8.4	42.2
18:0	50.5 ± 1.1	13.6	31.0 ± 0.9	9.8
20:0			16.3 ± 0.1	5.2
22:0	41.3 ± 1.3	11.1	34.8 ± 0.5	11.1
Total	370.8	100.0	314.7	100.0
(b) Acetone-eluted lipids				
16:1			18.8 ± 0.3	3.6
16:0	93.7 ± 2.6	13.1	48.0 ± 0.9	9.2
18:1	126.8 ± 2.9	17.8	119.5 ± 3.5	23.0
18:0	157.8 ± 5.5	22.1	12.0 ± 0.1	2.3
2-Me 18:1	111.5 ± 6.2	15.6	115.5 ± 4.6	22.2
20:0	61.5 ± 1.7	6.6	51.8 ± 1.7	9.9
22:0	163.0 ± 4.3	22.8	155.5 ± 5.1	29.8
Total	714.3	100.0	521.1	100.0
(c) Methanol-eluted lipids				
16:1	32.0 ± 1.3	5.0	28.0 ± 0.3	6.0
16:0	199.0 ± 6.8	30.9	130.0 ± 8.2	27.8
18:1	200.0 ± 6.1	31.0	105.8 ± 8.1	22.7
18:0	9.0 ± 0.3	1.4		
2-Me 18:1	58.0 ± 1.8	9.0	78.8 ± 1.0	16.9
10-Me 18:0	73.0 ± 2.6	11.3	23.0 ± 0.9	4.9
20:0	30.0 ± 2.1	4.7	37.0 ± 0.7	7.9
22:0	43.0 ± 1.2	6.7	64.3 ± 1.7	13.8
Total	644.0	100.0	466.9	100.0

The presence of docosanoic acid (22:0) indicate the presence of mycolic acids non-covalently-linked to arabinogalactan. These mycolates occur in mycobacteria in the form of monomycoloyl and dimycoloyl trehalose, which are well known components of the cell wall glycolipids (Daffe and Draper, 1998; Brennan and Nikaido, 1995). The increased amount of docosanoic acid detected in EMB-treated cells may result in effective inhibition of arabinogalactan biosynthesis which limits number of mycolate attachment sites in the cell wall skeleton. A high level of octadecenoic acid 18:1 (50% of fatty acids in EMB treated cells and only 34% in control cells) was another feature of the fatty acid profile of acetone-eluted lipids obtained from cells exposed to ethambutol. Unsaturated fatty acids increase lipid fluidity and thereby facilitate penetration of compounds through lipid layers.

The phospholipids eluted by methanol from free lipids zone are esterified by tuberculostearic acid (10-Me 18:0). In the EMB-treated samples the content of the tuberculostearic acid was 2 times lower than in control. Octadecenoic acid (18:1) in the EMB-treated preparation was distinctly lower comparing to control. Significant changes have been observed in the content of the 2-Me 18:1. In the presence of inhibitor, the quantity of this fatty acid was almost twice as high as in the control.

Discussion

It is generally accepted that the crucial permeability barrier in mycobacterial cells is the cell wall skeleton composed of peptidoglycan and arabinogalactan with covalently bound mycolic acids. The function of mycolic acids as the permeability barrier in mycobacteria has been confirmed by studies including direct measurements of the uptake of compounds by mycobacterial cells with altered mycolates content. An *M. tuberculosis* mutant strain with inactivated gene coding for antigen 85C, a protein associated with mycoloil-transferase activity, transferred 40% less mycolates on arabinogalactan than its parent strain. The reduction of cell wall-linked mycolic acids resulted in enhanced uptake of substrates by the mutant strain (Jackson *et al.*, 1999). Similar results were obtained for the *csp-1* inactivated mutant of *Corynebacterium glutamicum*, deficient in PS1 protein which is similar to the antigen 85 complex of *M. tuberculosis*. This mutant containing only 50% of cell wall-bound corynemycolates exhibited considerably higher glycerol and acetate uptake what confirmed that the amount of cell wall-linked mycolates is important for the cell envelope permeability (Puech *et al.*, 2000). The permeability of the cell wall can be increased using inhibitors of its biosynthesis. We have previously shown that glycine enhanced sitosterol biotransformation activity by disorganization of peptidoglycan and partial disintegration of mycolic acids (Sedlaczek *et al.*, 1999). On the other hand the enhanced quantity of the unsaturated fatty acids increased the fluidity of the free lipids layer resulting in increased penetration of steroid compounds through the cell wall (Korycka-Machała *et al.*, 2001; Rumijowska *et al.*, 1997; 2000).

In this study we found that ethambutol allows for increased penetration of sitosterol through cell wall barrier by disorganization of cell wall skeleton and free lipids. We have previously shown, that in the presence of ethambutol susceptibility of *M. vaccae* to antibiotics was distinctly higher (Korycka-Machała *et al.*, 2005). Ethambutol inhibits the biosynthesis of arabinogalactan interfering with both the arabinan and galactan segments (Xin *et al.*, 1999; Sirgel *et al.*, 2000). EMB acting by inhibition of arabinofuranosyl transferases responsible for the glycosylation steps in the biosynthesis of arabinogalactan and lipoarabinomannan (Belanger *et al.*, 1996; Häusler *et al.*, 2001; Pathak *et al.*, 2004). It was reported that EMB disintegrates arabinogalactan of *M. smegmatis* resulting in decreasing in the number of attachment sites for the mycoloyl residues (Takayama and Kilburn, 1989; Daffe and Draper, 1998; Ramaswamy and Musser, 1998).

Very recently Radmacher *et al.* (2005) demonstrated that *Corynebacterium glutamicum* treated with EMB decreased arabinan deposition in the arabinogalactan resulting in reduced mycolic acid content. This suggests that similar mechanisms of EMB toxicity exist in both *C. glutamicum* and *Mycobacterium* strains.

We found decreased amount of mycolic acids in the cell wall skeleton exposed to ethambutol and increased amount of mycolic acids in the glycolipids fraction and post-cultures medium, which likely resulted from lower number of mycolate attachment sites in arabinogalactan. This shift of mycolic acids in the cell envelope enhanced permeation of the hydrophobic compounds through the cell wall. Therefore, the direct effect of ethambutol for biosynthesis of arabinogalactan is followed by disorganization of the cell wall outer layer, its loss of integrity and changes of free lipids composition. The observed effect of increased permeability results likely from all the above changes.

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