International Journal of Cosmetic Science, 2008, **30**, 339–345

Cocoa polyphenols and their influence on parameters involved in *ex vivo* skin restructuring

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Received 22 November 2007, Accepted 7 May 2008

Keywords: cocoa butter, cocoa polyphenols, *ex vivo* model, human skin explants

Synopsis

Polyphenols in general are compounds that are known to promote health and have a preventive effect against various chronic diseases. The influence of cocoa polyphenols on skin, however, has scarcely been studied from a histological point of view. The aim of this study is to assess the influence of cocoa polyphenols on several indicators of skin elasticity and skin tonus, namely, glycosaminoglycans and collagen I, III and IV. This was carried out by using a model of *ex vivo* human skin explants maintained in survival, on which a cocoa polyphenol extract was applied. After processing by standard histological techniques (fixation, paraffin embedding, sectioning, staining, immunostaining and microscopical observation), the influence of cocoa polyphenols on the evaluated parameters was quantified by image analysis. The results obtained show that cocoa polyphenols exhibit a positive action on the parameters assessed, and the dose at which they improve the most parameters associated with skin tonus and elasticity was determined. Their activity was compared with a commercially available product, and the results obtained show that their efficacy is equivalent. Moreover, an enhancing effect of cocoa butter on activity of cocoa polyphenol was highlighted. Now that the properties of cocoa polyphenols on *ex vivo* skin restructuring parameters have been assessed, the next step could include their evaluation *in vivo*.

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Résumé

Les polyphénols sont des composés connus pour avoir un effet bénéfique sur la santé, et une action préventive sur de nombreuses maladies chroniques. L'influence des polyphénols de cacao sur la peau, en revanche, a été peu étudiée d'un point de vue histologique. L'objectif de cette étude est d'évaluer l'influence des polyphénols de cacao sur plusieurs marqueurs de l'élasticité et du tonus cutané, c'est-à-dire les glycosaminoglycanes et les collagènes I, III et IV. L'étude a été réalisée sur un modèle *ex vivo* d'explants de peau humaine maintenus en survie, sur lesquels a été appliqué un extrait de polyphénols de cacao. Après traitement par les techniques histologiques classiques (fixation, imprégnation en paraffine, coupe, coloration, immunomarquage et observation microscopique), l'influence des polyphénols de cacao sur les paramètres mesurés a été quantifiée par analyse d'image. Les résultats obtenus montrent que les polyphénols de cacao présentent une activité sur les paramètres évalués, et la dose à laquelle ils améliorent le mieux les paramètres cutanés a pu être déterminée. L'activité des polyphénols a été comparée à celle d'une crème cosmétique disponible dans le commerce, et les résultats montrent que leur efficacité est équivalente. Par ailleurs, un effet amplificateur du beurre de cacao sur l'activité des polyphénols de cacao a pu être mis en évidence. Les propriétés des polyphénols de cacao sur la restructuration de la peau ayant été évaluées *ex vivo*, il faudrait désormais poursuivre les investigations *in vivo*, ainsi que sur d'autres paramètres.

Introduction

Several thousands of molecules with a polyphenol structure have been identified in higher plants and several hundred are found in edible plants. These molecules are secondary metabolites of plants and generally involved in defence against external stressors like ultraviolet radiation or aggression by pathogens [1]. Polyphenols may be further classified into different groups as a function of the number of aromatic rings and the structural elements that bind these rings together. Distinctions are made between flavonoids, non-flavonoids and phenolic acids (see Fig. 1), with the first group being the largest one with more than 2000 known compounds.

Cocoa and cocoa-derived products are rich in polyphenols and particularly in flavonoids, a class of compounds that occur in a wide variety of fruits, vegetables, teas and red wines. It is well documented that cocoa and cocoa products like chocolate are among the richest sources of polyphenols [2]. In addition, cocoa has been described to be rich in a particular subgroup of flavonoids named flavanols (flavan-3-ols). The flavanols are present as the monomers epicatechin and catechin or as oligomers of epicatechin and/or catechin called procyanidins.

An increasing body of evidence supports the concept that dietary intake of polyphenols promotes health and attenuates or delays the onset of various diseases, including cardiovascular diseases, cancer and other chronic diseases. Flavanols in cocoa and cocoa products exert some beneficial vascular effects [3, 4], reduce the risk for cardiovascular morbidity and mortality [5] and cancer [6] and may contribute to the prevention of neurodegenerative diseases and diabetes mellitus [7]. Heinrich *et al.* [8] found that long-term ingestion of cocoa flavanols contributes to photoprotection against ultraviolet (UV) irradiation, increases dermal blood flow and skin thickness, improves skin density and moisture and influences significantly the skin structure and roughness. In another study from the same research group [9], an increase in dermal blood flow and oxygen saturation of haemoglobin was detected within 2 h after ingestion of a single dose of flavanol-rich cocoa. Cocoa butter is already widely used as an active excipient in skin care products; however, additional experiments are needed to fully explore the potential of cocoa polyphenols, particularly concerning moisturizing and anti-ageing.

In this study, we intend to explore further the activity of cocoa polyphenols on skin structure by histological techniques through the examination of parameters such as general skin morphology, influence on the expression of glycosaminoglycans (GAGs) and influence on the expression of three collagen types. Neutral GAGs close to the dermal-epidermal junction (DEJ) are known to be rich in growth factors; acidic GAGs in the epidermis and papillary dermis are essentially constituted of hyaluronic acid which is involved in skin moisture; they both constitute the major part of the extra cellular matrix. As for collagen, types I and III are components of the dermal fibrillary structure; type IV collagen is an important connection component of the DEJ. *In vivo*, GAGs are involved in skin elasticity and moisture [10–12] and collagens are responsible for skin tonus [13].

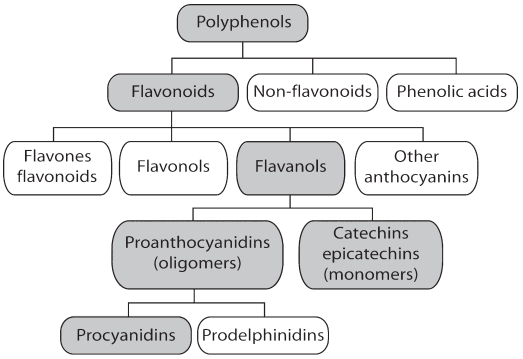

Figure 1 Classification hierarchy of polyphenols with epicatechin, catechin, and the procyanidins being the predominant class of polyphenols in cocoa (indicated in grey) [2].

Materials and methods

Tested products

The cocoa polyphenol extract used is the Acticoa™ cocoa (Barry Callebaut, Zürich, Switzerland), a natural material prepared from selected cocoa beans that are naturally rich in polyphenols. On top of this selective sourcing, a special process known as the Acticoa™ process is applied to preserve the maximum amount of cocoa polyphenols in the cocoa bean. These beans are then used as a raw material for the extraction to produce a cocoa polyphenol extract, which has 45% polyphenol content, with 5.17% epicatechin and 0.75% cate-

chin. The cocoa butter used was Mycryo® (Barry Callebaut), a form of cocoa butter reduced to a dry powder by freezing at a temperature of −65°C. It was restored to a paste texture by gentle mixing. Cocoa butter in general does not contain polyphenols.

Ex vivo model

For each experiment, explants were prepared from abdominal plasties of Caucasian women aged 39–68 years (three donors). Our experience in the domain of explants survival has shown that on over 550 different donors, the response to the treatments is noticeably similar. The subcutaneous fat was removed from the skin and explants of around 10 mm in diameter were cut out using a circular scalpel. The explants were then put in survival in classical cell culture conditions, in 2 mL BIO-EC's explant medium (BEM; Laboratoire BIO-EC, Longjumeau, France), half of which was renewed every other day. BEM is a culture medium specifically engineered for explants survival, the composition of which remains undisclosed for confidentiality reasons. The explants were maintained in survival for up to 12 days. Each batch comprised six explants (three for each sampling time) and each experiment comprised a control batch of nine explants (three for each sampling time, and three for the T0 control). The products were tested by topical application of 2 mg on the skin explants, spread with a small spatula.

Histology

On the sampling day, samples from each batch were withdrawn and cut in two parts: one half was frozen at −75°C; the other half was fixed in ordinary Bouin's solution (7.5% formaldehyde, 5% acetic acid aqueous solution saturated with picric acid) for 48 h, then dehydrated and impregnated in paraffin (Paraplast X-tra; McCormick Scientific, St Louis, MO, U.S.A) using a Leica 1020 dehydration automat (Leica, Nussloch, Germany). The impregnated samples were then embedded using a Leica EG 1160 embedding station. Five micrometre thick sections were made using a Leica RM 2125 Minot-type microtome, and the sections were then bonded on histological glass slides for staining. Frozen samples were cut into 7-μm-thick sections using a Leica CM 3050 cryostat. Sections were then bonded on silanized glass slides (SuperFrost Plus; Menzel Gläser, Braunschweig, Germany) for immunostaining.

Staining – immunostaining

All specific dyes used in the various stainings were prepared in the laboratory from reagents available from regular histology suppliers. All chemicals used were of the highest purity available. The cellular viability and general skin morphology of each batch was assessed after staining deparaffinized sections according to Masson's trichrome, Goldner variant, using a ST 4040 Leica staining automat. GAGs were visualized after alcian blue-PAS staining (Mowry staining).

Immunostaining of type I and type IV collagen was carried out using, respectively, an anti-collagen-I polyclonal antibody (PS047 from Monosan, Uden, the Netherlands) and an anti-collagen-IV polyclonal antibody (1330–01 from SBA, Birmingham, AL, USA), with a biotin/streptavidin enhancement system and revealed by fluorescein isothiocyanate. Nuclei were post-stained with propidium iodide. Immunostaining of type III collagen was carried out using an anti-collagen-I polyclonal antibody (1340–01 from SBA), with a biotin/streptavidin enhancement system and revealed by diaminobenzidine. Nuclei were post-stained by Masson's hemalum.

Microscopical observations

The microscopical observations were carried out using a Leica DMLB microscope with a 40× objective. Photos were taken with a tri-CCD DXC 390P Sony camera (Sony, Tokyo, Japan) and stored using the Leica IM1000 data storing software.

Image analysis

An image analysis was carried out using the Leica Qwin software on five microscopical fields (63× magnification). The photos were digitized and the staining was detected by intensity level selection. The area of interest was then selected (either the papillary dermis or a 10-μm thick area under the DEJ). The surface occupied by the staining in the area of interest was then measured and expressed as a percentage of surface. For type I or type III collagen, the area of interest was the papillary dermis and for GAGs or type IV collagen, it was a thin strip under the DEJ.

Results and discussion

Assessment of the activity

The activity of cocoa polyphenols on skin structure was assessed by topical application of either 1.5% cocoa polyphenols blended with cocoa butter (CPP) or cocoa butter alone (CB) on day 0, day 1, day 2, day 5, day 7 and day 9. The skin explants were prepared from a 68-year-old donor. Three explants from each batch (CPP, CB and untreated control U) were sampled on day 5 and day 12. The explants were then processed as described previously and the various parameters presented above were evaluated.

The results show that after 5 days, cocoa polyphenols induce a slight increase in epidermal thickness as well as an increase in collagen density in the papillary dermis. The increase in collagen density is confirmed after 12 days. Regarding GAGs, no change compared with the control batch is observed for the CB batch, whereas the CPP batch exhibits a slight increase in the amount of GAGs observed in the papillary dermis. A small increase in type I collagen expression is observed after 5 days for the CPP batch; after 12 days the increase is clear, whereas the CB batch displays only a moderate increase. Concerning both type III and type IV collagen, a clear increase is observed on day 5 for the CPP batch; after 12 days the increase is even more marked. In the CB batch, for all three collagen subtypes, the increase observed after 5 days is lesser than that of the CPP batch; after 12 days the increase is similar to that of the CPP batch.

These results demonstrate that cocoa polyphenols, when blended with cocoa butter, have a positive influence on the measured parameters as early as 5 days after the start of the treatment. The activity of cocoa butter alone is similar to the above-mentioned effect, but it takes longer for it to exert the same influence on the skin structure. As cocoa butter does not contain any polyphenols, there is strong evidence that cocoa polyphenols alone can induce a rapid improvement in parameters involved in skin tonus and elasticity.

Determination of the effective dose

The next experiment consisted in determining the dose at which cocoa polyphenols have the best restructuring activity. This was performed by topical application of cocoa polyphenols at the concentration of 0.125%, 0.25%, 0.50%, 0.75% and 1.0%, blended with cocoa butter. The skin explants were prepared from a 43-year-old donor. The applications took place on day 0, day 1, day 2, day 4, day 6, day 8 and day 10, and three explants from each batch were sampled on day 6 and day 11. The explants were then processed as described previously, and the results show that every concentration of polyphenols tested was without harmful effects. This implies that overall product tolerance is very good. The observation of the general morphology shows that on the explants treated with CB alone, the epidermal structure is close to that observed on the control explants. On the other hand, on the explants treated with CPP 0.75, the epidermal thickness is clearly increased (Fig. 2).

After examination of the general morphology, further investigations (staining of GAGs and immunostaining of type I, III and IV collagen) were focussed on the explants treated with the various concentrations of polyphenols for 11 days. After 11 days, the concentrations of cocoa polyphenols induce an increase in GAG expression in the following order: 0.75 (very clear) > 0.50 (clear) > 0.125 (slight) > 1.0 (moderate) > 0.25 (very moderate). The ranking for type I collagen is 0.75 (very clear) > 0.25 = 0.50 = 0.125 (clear) > 1.0 (moderate); for type III collagen, 0.50 = 0.25 = 0.125

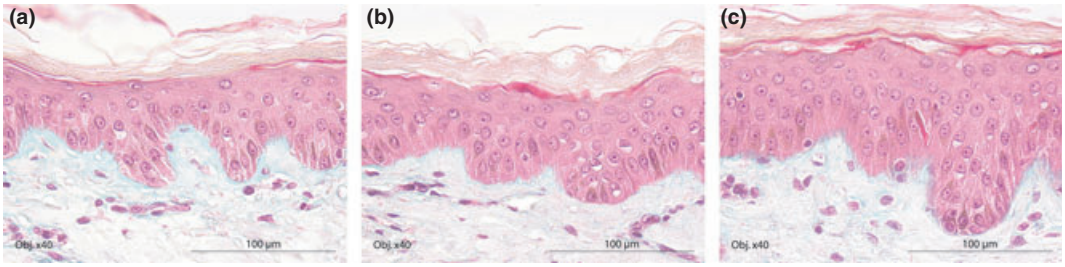

Figure 2 General morphology. (a) Untreated explants (D 11), (b) explants treated with CB alone (D 11), (c) explants treated with 0.75% CPP (D 11). CPP, cocoa polyphenols blended in cocoa butter; CB, cocoa butter alone.

(moderate, moderately dense) > 0.75 = 1.0 (moderate, slightly dense); and for type IV collagen, 0.25 (very clear) > 0.75 = 0.50 = 1.0 = 0.125 (clear). In conclusion, the concentration at which cocoa polyphenols blended with cocoa butter induce the best overall skin restructuring is 0.75% or 0.50%. The pictures of the explants treated with the polyphenols concentration that exhibit the best response for each parameter assessed are presented in Fig. 3.

Comparison with a commercially available product

To assess the efficacy of the polyphenol-containing formulas in comparison with commercially available products, an anti-ageing cream (Future Perfect; Estee Lauder, New York, NY, USA) was tested in parallel with the various concentrations assessed in the previous experiment. The cream was applied on day 0, day 1, day 2, day 4, day 6, day 8 and day 10, and samples were removed on day 6 and day 11. The explants were then processed as described above. After 11 days, the increase in GAGs expression is similar to that of explants treated with 1.0% CPP (moderate), in the case of type I collagen, it corresponds to that of 1.0% CPP (moderate), for type III collagen to 0.125%, 0.25% and 0.50% CPP (moderate, moderately dense) and for type IV collagen to 0.25% CPP (very clear).

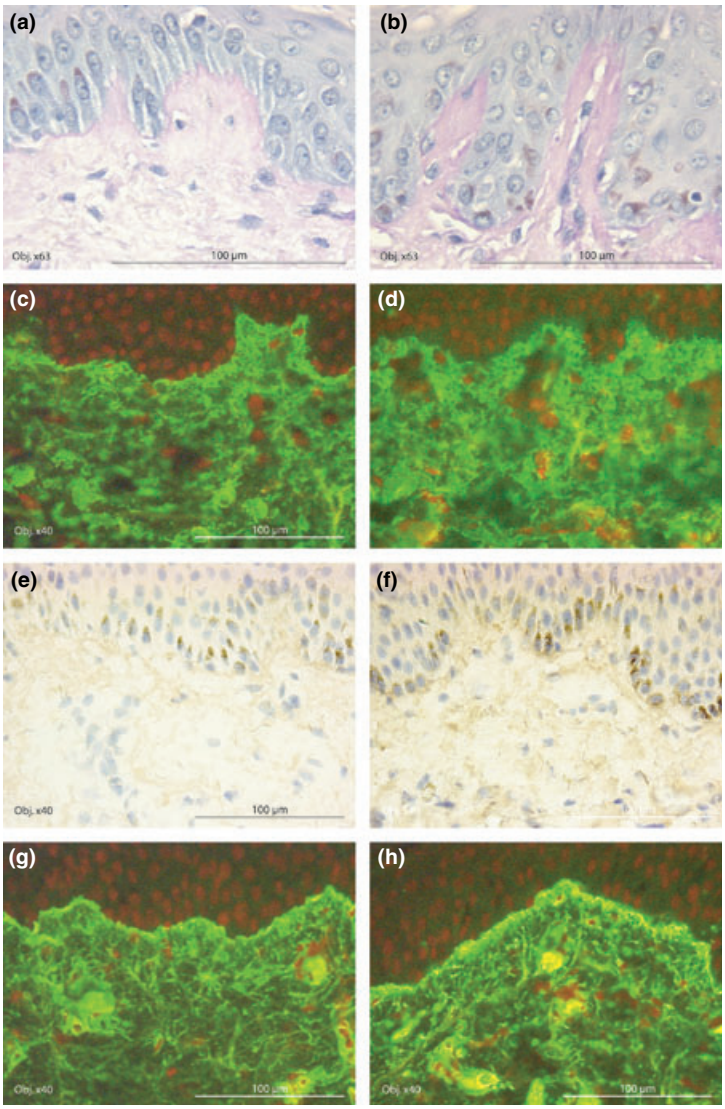

Figure 3 Pictures of stainings and immunostainings. (a) GAGs, untreated explants (D 11), (b) GAGs explants treated with 0.75% CPP (D 11), (c) collagen I, untreated explants (D 11), (d) collagen I, explants treated with 0.75% CPP (D 11), (e) collagen III, untreated explants (D 11), (f) collagen III, explants treated with 0.50% CPP (D 11), (g) collagen IV, untreated explants (D 11) and (h) collagen IV, explants treated with 0.25% CPP (D 11). GAGs, glycosaminoglycans; CPP, cocoa polyphenols blended in cocoa butter.

Table I Image analysis data

	GAGs			Collagen I			Collagen III			Collagen IV		
	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%
Untreated control	15.5	6.7	–	20.7	8.0	–	31.7	13.8	–	20.1	6.4	–
CPP (0.75%)	46.3	8.2	198	41.2	10.4	99	38.9	5.3	23	29.5	7.6	47
CPP (0.50%)	38.1	2.9	145	28.4	9.5	38	50.9	7.8	61	32.0	4.1	60
Estee Lauder (Future Perfect)	47.5	5.2	206	17.0	2.5	–18	54.3	11.3	72	42.2	10.4	111

GAGs, glycosaminoglycans; CPP, 1.5% cocoa polyphenols blended with cocoa butter.

Image analysis

An image analysis was carried out on the sections from the following batches sampled on day 11: untreated control (U), CPP 0.50% (CPP 0.50), CPP 0.75% (CPP 0.75) and reference cream (RC). The parameters assessed were percentage of surface occupied by type I or type III collagen in the papillary dermis and percentage of surface occupied by GAGs or type IV collagen close to the DEJ. The raw data of CPP batches are presented in Table I as mean percentage of surface, standard deviation and percentage variation and compared with the untreated control. A diagrammatical representation is shown in Fig. 4.

The qualitative results obtained in the previous experiments are substantiated by these results. A very clear increase in GAGs expression is observed for explants treated with CPP 0.75% (+198%) and CPP 0.50% (+145%). As for those treated with the RC, the increase (+206%) is same as that of CPP 0.75%. Concerning type I collagen, the only sample that is significantly different from the untreated control is the CPP 0.75% batch (+99%). As for type III and type IV collagen, only samples treated with CPP 0.50% (+60% and +59% respectively) or the RC (+71% and +110%) exhibit a significant increase compared with the control.

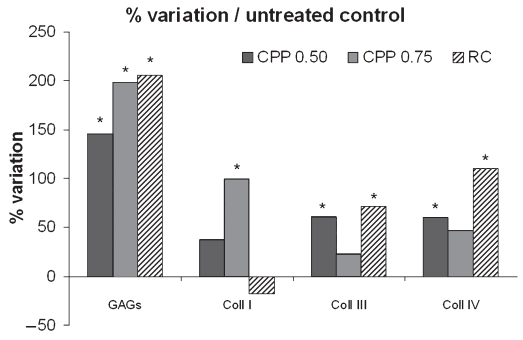

Figure 4 Variation of the expression of GAGs, type I, type III and type IV collagen as a function of the product tested. *Indicate a statistical difference from the control, $P < 0.05$. GAGs, glycosaminoglycans; CPP, cocoa polyphenols blended in cocoa butter; RC, reference cream.

Enhancing effect

The boosting effect of cocoa butter on activity of cocoa polyphenols was assessed by topical application of formulas containing 0.5% cocoa polyphenols (CPP), 5% cocoa butter (CB) or both (CPP + CB). The skin explants were prepared from a 39-year-old donor. The formulas were applied topically on day 0, day 1, day 2, day 4, day 6, day 8 and day 10, and samples were removed on day 5 and day 11. The formulas used were manufactured on the basis of the list of ingredients presented in Table II.

The parameters assessed were the general skin morphology and expression of GAGs, type I and type IV collagen. There is a moderate increase in GAGs expression near the DEJ after 5 days of treatment for all three batches. After 11 days, however, clear differences in GAGs expression for the CPP + CB batch appear, whereas the increase is moderate for either CPP or CB alone. Concerning type I collagen, after 5 days, both CPP and CB batches show a slight increase in its expression, whereas the CPP + CB batch displays a clear increase. However, after 11 days, all three batches display the same very clear increase in type I collagen expression. As for type IV collagen, after 5 days of treatment, the increase in expression is observed only for the CB batch. After 11 days, there is a slight increase for the CPP batch, a clear increase for the CB batch, and the CPP + CB batch shows a very clear increase in its expression. Con-

Table II List of ingredients

INCI Name	%
Water qsp	100
Mineral oil	6.00
PPG-15 stearylether	6.00
Theobroma cocoa seed butter	5.00
Cyclopentasiloxane	4.00
Glycerine	4.00
Steareth-2	3.00
Steareth-21	3.00
Polyacrylamide	0.80
Theobroma cocoa seed extract	0.50
Phenoxyethanol	0.44
C13-14 isoparaffin	0.30
Xanthan gum	0.20
Laureth-7	0.10
Tetrasodium EDTA	0.10
Methyl paraben	0.09
Butyl paraben	0.02
Ethyl paraben	0.02
Propyl paraben	0.01
Isobutyl paraben	0.01

PPG, polypropyleneglycol; EDTA, ethylene-diaminetetraacetate.

sequently, the enhancing effect of cocoa butter on cocoa polyphenols is highlighted after 5 days in the case of type I collagen or after 11 days in the case of GAGs and type IV collagen.

Conclusion

In this study, we have shown that cocoa polyphenols exhibit a positive effect on skin structure when applied for at least 5 days. Cocoa butter also exhibits an activity, but its onset of action is longer (12 days). The dose at which cocoa polyphenols – when blended with cocoa butter – improve the most *ex vivo* parameters associated with skin tonus and elasticity is between 0.50% and 0.75%. At such doses, the influence on parameters such as GAGs and collagen expression is comparable (or better, in the case of collagen I) than a commercially available anti-ageing cream. Moreover, when applied in conjunction with cocoa polyphenols, cocoa butter exhibits an enhancing effect on the parameters assessed. In next stage, the exploration of cocoa polyphenols properties could include the evaluation of their influence on skin moisturizing and restructuring *in vivo*, assessment of protective effect against UV exposure and biochemical mechanism of action.

Acknowledgement

This study was funded jointly by Laboratoire BIO-EC and Barry Callebault.

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