

1 Supplemental information

2 **Supplementary Equation 1: Postulated mathematical models associated with each type of variables**

3 **are:**

- 4 • Qualitative factor X1: $Y_{\text{qual.}} = b_0 + b_{1A} X_{1A} + b_{1B} X_{1B}$
- 5 • Quantitative factors X2, X3, X4 : $Y_{\text{quant.}} = b_0 + b_2 X_2 + b_3 X_3 + b_4 X_4 + b_{22} X_2^2 + b_{33} X_3^2 + b_{44} X_4^2 + b_{23}$
6 $X_2 X_3 + b_{24} X_2 X_4 + b_{34} X_3 X_4$

7 To take into consideration the interaction effect between the qualitative and quantitative factors, a
8 product model was postulated:

9 $Y = Y_{\text{qual}} \times Y_{\text{quant.}} = b_0 + b_{1A} X_{1A} + b_{1B} X_{1B} + b_{1C} X_{1C} + b_2 X_2 + b_{21A} X_2 X_{1A} + b_{21B} X_2 X_{1B} + b_{21C} X_2 X_{1C} + b_3 X_3 + b_{31A} X_3 X_{1A} +$
10 $b_{31B} X_3 X_{1B} + b_{31C} X_3 X_{1C} + b_4 X_4 + b_{41A} X_4 X_{1A} + b_{41B} X_4 X_{1B} + b_{41C} X_4 X_{1C} + b_{22} X_2^2 + b_{221A} X_2^2 X_{1A} + b_{221B} X_2^2 X_{1B} + b_{221C} X_2^2 X_{1C} +$
11 $b_{33} X_3^2 + b_{331A} X_3^2 X_{1A} + b_{331B} X_3^2 X_{1B} + b_{331C} X_3^2 X_{1C} + b_{44} X_4^2 + b_{441A} X_4^2 X_{1A} + b_{441B} X_4^2 X_{1B} + b_{441C} X_4^2 X_{1C} + b_{23}$
12 $X_2 X_3 + b_{231A} X_2 X_3 X_{1A} + b_{231B} X_2 X_3 X_{1B} + b_{231C} X_2 X_3 X_{1C} + b_{24} X_2 X_4 + b_{241A} X_2 X_4 X_{1A} + b_{241B} X_2 X_4 X_{1B} + b_{241C} X_2 X_4 X_{1C} + b_{34}$
13 $X_3 X_4 + b_{341A} X_3 X_4 X_{1A} + b_{341B} X_3 X_4 X_{1B} + b_{341C} X_3 X_4 X_{1C}$
14

15 To estimate the 40 coefficients of this model and predict the response in the whole domain with good
16 quality a D-optimal design of experiments, was built from the Fedorov algorithm (Fedorov, V.V. Theory of
17 Optimal Experiments, Academic Press, N.Y., 1972). This design consisted of 56 experiments.

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19 **Supplementary Equation 2: Overall desirability function (D)**

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$$D = \sqrt[25]{d_1^4 \times d_2^3 \times d_3^5 \times d_4^4 \times d_5^3 \times d_6^1 \times d_7^1 \times d_8^2 \times d_9^2}$$

21 The optimal condition was multidimensional space defined as the largest volume around the point
22 where the global desirability was maximum, in practice different from 0, with the guarantee that the
23 objectives were satisfied with a probability of 95% (Quality by Design concept)(Sangshetti, J.N. et al.
24 *Arab. J. Chem.* **10**, S3412-S3425 (2017).

25 **Supplementary Table 1: Coefficients for HAI response models**

Formulation	SE	SE	AF	AF	AF	LSQ	LSQ	LSQ	AI	AI	AI
Day	35	35	21	35	35	21	35	35	21	35	35
Sample	Serum	IW	Serum	Serum	IW	Serum	Serum	IW	Serum	Serum	IW
Constant	3.51	0.21	2.14	3.62	2.06	2.49	3.72	2.30	2.55	4.09	2.12
CfaEB	0.12	0.55	-0.15	-0.05	0.01	0.24	0.10	0.04	0.10	0.15	0.09
SLA	0.09	0.21	0.00	-0.06	0.03	-0.02	0.02	-0.02	0.01	-0.03	0.06
dmLT	-0.05	0.55	0.03	0.02	-0.05	-0.02	0.00	-0.08	-0.09	-0.04	-0.13
CfaEB * CfaEB	0.05	0.21	0.05	-0.16	0.09	-0.08	0.09	0.08	0.05	0.05	-0.01
SLA * SLA	0.04	0.55	-0.43	0.02	0.10	-0.10	-0.14	-0.02	-0.02	-0.09	-0.09
dmLT * dmLT	0.01	0.21	0.40	0.11	0.00	0.10	0.07	0.01	-0.09	-0.02	0.16
CfaEB * SLA	-0.02	0.55	0.12	-0.05	0.02	-0.03	-0.04	0.02	-0.02	0.05	-0.02
CfaEB * dmLT	-0.01	0.21	-0.15	0.05	-0.02	0.04	-0.05	-0.03	-0.04	0.03	0.02
SLA * dmLT	0.04	0.55	0.09	-0.02	-0.07	-0.02	0.06	-0.07	0.01	0.00	-0.03
Adjusted R ²	0.39	N/A	0.48	0.17	N/A	0.77	0.45	0.83	0.64	0.83	0.15

26 Student's T test; Bold: p-value <0.05

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28 **Supplementary Table 2: Coefficients for LT neutralization response models**

Formulation	SE	AF	AF	LSQ	LSQ	AI	AI
Day	21	21	35	21	35	21	35
Sample	Serum	Serum	Serum	Serum	Serum	Serum	Serum
Constant	1.89	1.63	3.64	1.39	3.08	1.57	3.35
CfaEB	0.03	-0.09	0.05	-0.01	-0.06	0.03	-0.03
SLA	0.13	-0.03	0.01	0.10	0.10	0.00	0.04
dmLT	0.28	-0.21	0.06	0.46	0.57	0.39	0.69
CfaEB * CfaEB	0.19	0.11	-0.20	-0.12	-0.06	-0.11	-0.01
SLA * SLA	0.15	0.02	0.21	0.05	-0.16	0.12	0.04
dmLT * dmLT	-0.11	-0.01	0.02	0.22	0.13	0.00	-0.13
CfaEB * SLA	-0.06	-0.12	0.01	0.02	-0.11	-0.10	-0.10
CfaEB * dmLT	-0.23	-0.20	-0.14	0.10	0.09	-0.09	-0.01
SLA * dmLT	0.00	-0.02	0.06	0.08	0.05	0.03	0.03
Adjusted R ²	0.88	0.11	n.d.	0.96	0.98	0.65	0.96

29 Student's T test; Bold: p-value <0.05

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31 **Supplementary Table 3: Coefficients for IW IgG response models**

Formulation	SE	AF	AF	LSQ	LSQ	AI	AI
Antigen	CfaEB	dmLT	CfaEB	dmLT	CfaEB	dmLT	CfaEB
Sample	IW	IW	IW	IW	IW	IW	IW
Constant	3.37	3.24	3.70	3.88	4.31	2.93	3.93
CfaEB	0.07	-0.04	0.09	-0.02	0.13	-0.03	0.20
SLA	0.00	0.03	0.20	0.12	0.09	0.08	0.03
dmLT	0.00	0.25	-0.15	0.38	0.10	0.46	-0.07
CfaEB * CfaEB	0.06	-0.01	-0.14	0.10	0.04	0.04	-0.01
SLA * SLA	0.11	0.09	0.33	-0.14	-0.15	-0.14	-0.09
dmLT * dmLT	-0.13	-0.04	-0.11	0.01	-0.06	-0.08	-0.01
CfaEB * SLA	0.01	0.04	-0.02	-0.05	-0.04	0.02	0.05
CfaEB * dmLT	0.00	-0.05	0.06	-0.02	0.01	-0.03	-0.01
SLA * dmLT	0.05	0.02	0.01	-0.13	-0.14	0.03	0.12
Adjusted R ²	n.d.	0.65	0.82	0.92	0.77	0.86	0.36

32 Student's T test; Bold: p-value <0.05

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34 **Supplementary Table 4: Coefficients for ASC response models**

Formulation	SE	AF	AF	LSQ	LSQ	AI	AI
Antigen	dmLT	dmLT	CfaEB	dmLT	CfaEB	dmLT	CfaEB
Sample	ASC	ASC	ASC	ASC	ASC	ASC	ASC
Constant	1.57	1.18	1.97	1.69	2.33	1.20	1.93
CfaEB	-0.02	0.00	0.06	-0.04	-0.07	0.04	0.13
SLA	0.05	0.03	0.00	-0.02	0.11	0.07	-0.09
dmLT	0.39	0.37	0.27	0.26	0.19	0.35	0.22
CfaEB * CfaEB	-0.11	-0.23	0.01	-0.03	-0.09	0.13	0.09
SLA * SLA	-0.03	0.21	0.13	-0.10	-0.06	-0.02	-0.02
dmLT * dmLT	-0.06	0.28	0.10	-0.04	-0.06	-0.08	-0.01
CfaEB * SLA	-0.03	-0.04	-0.03	0.12	0.07	-0.02	-0.05
CfaEB * dmLT	0.03	-0.04	0.00	0.00	0.03	0.01	-0.01
SLA * dmLT	-0.01	0.00	0.04	0.09	-0.06	-0.11	0.03
Adjusted R ²	0.79	0.48	n.d.	0.47	0.58	0.89	0.60

35 Student's T test; Bold: p-value <0.05

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37 **Supplementary Table 5: Design of experiments used to establish the RSM for each immune response**

Group	CfaEB (μg)	SLA (μg)	dmLT (μg)	Formulation
1	0.3	0.05	0.03	SE
2	10	0.05	0.03	SE
3	1.7	0.5	0.03	SE
4	0.3	5	0.03	SE
5	10	5	0.03	SE
6	1.7	0.05	0.17	SE
7	0.3	0.5	0.17	SE
8	10	0.5	0.17	SE
9	1.7	5	0.17	SE
10	0.3	0.05	1	SE
11	10	0.05	1	SE
12	1.7	0.5	1	SE
13	0.3	5	1	SE
14	10	5	1	SE
15*	1.7	0.5	0.17	SE
16*	1	0.1	1	SE
17	0.3	0.05	0.03	AF
18	10	0.05	0.03	AF
19	1.7	0.5	0.03	AF
20	0.3	5	0.03	AF

21	10	5	0.03	AF
22	0.3	0.05	0.17	AF
23	10	0.5	0.17	AF
24	1.7	5	0.17	AF
25	0.3	0.05	1	AF
26	1.7	0.05	1	AF
27	10	0.05	1	AF
28	0.3	0.5	1	AF
29	0.3	5	1	AF
30	10	5	1	AF
31*	1.7	0.5	0.17	AF
32*	4.2	0.2	0.4	AF
33	0.3	0.05	0.03	LSQ
34	10	0.05	0.03	LSQ
35	10	0.5	0.03	LSQ
36	0.3	5	0.03	LSQ
37	1.7	5	0.03	LSQ
38	10	5	0.03	LSQ
39	1.7	0.05	0.17	LSQ
40	0.3	0.5	0.17	LSQ
41	10	5	0.17	LSQ
42	0.3	0.05	1	LSQ
43	10	0.05	1	LSQ
44	1.7	0.5	1	LSQ
45	0.3	5	1	LSQ
46	10	5	1	LSQ
47*	1.7	0.5	0.17	LSQ
48*	0.7	3	0.7	LSQ
49	0.3	0.05	0.03	Alum
50	10	0.05	0.03	Alum
51	1.7	0.5	0.03	Alum
52	0.3	5	0.03	Alum
53	10	5	0.03	Alum
54	1.7	0.05	0.17	Alum
55	0.3	0.5	0.17	Alum
56	10	0.5	0.17	Alum
57	1.7	5	0.17	Alum
58	0.3	0.05	1	Alum
59	10	0.05	1	Alum
60	1.7	0.5	1	Alum
61	0.3	5	1	Alum
62	10	5	1	Alum
63*	1.7	0.5	0.17	Alum

	64*	1	0.1	1	Alum
38	* test points				
39					
40					

41 Glossary / Definition

42	AF	aqueous formulation
43	Al	aluminium oxyhydroxide
44	ASC	antibody secreting cells
45	cAMP	cyclic adenosine monophosphate
46	CF	colonization factor
47	CS	coli surface
48	CFA/I	colonization factor antigen I
49	CfaEB	colonization factor antigen fimbrial subunit E and B fusion
50	dmLT	double mutated heat-labile toxin
51	DoE	design of experiment
52	ETEC	enterotoxigenic <i>Escherichia coli</i>
53	HAI	haemagglutination
54	IgG	immunoglobulin G
55	IM	intramuscular
56	IW	intestinal washes
57	LSQ	liposomal Quillaja saponaria-21
58	LT	heat-labile toxin
59	SE	stable emulsion
60	SLA	second-generation lipid adjuvant
61	ST	stable toxin
62	TLR	Toll-like receptor
63	TLR4	Toll-like receptor 4 ligand
64	Linear effect	the effect of a factor is linear when the response evolves in a rectilinear way with regard
65		to this factor

66 Square effect the effect of a factor is squared when the response evolves in a parabolic way (that is
67 "shape of bell") when this factor evolves

68 Interaction we speak about interaction between two factors when the effect on the response
69 related to one of the factors depends on the level of the other factor

70 Significance: the significance of the statistical tests was estimated at a 5 % level (a P-value lower than
71 5 % or 0.05 corresponds to a significant test)