Research Article

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Impact of organic and conventional carrots on intestinal and peripheral immunity†

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Abstract

BACKGROUND: Studies on health effects of organic (ORG) products are still limited and often contradictory. We have investigated the impact of ORG and conventional (CV) carrots from two consecutive harvest years on mouse peripheral and intestinal immunity.

RESULTS: Danish carrots (*Bolero* variety) were grown in three ORG (O1, O2 and O3) and one CV cropping system (D-CV). Italian carrots (*Maestro* and *Excelso* varieties) were grown in one ORG and one CV field for each variety. Immune phenotypes of blood, spleen and intestinal lymphocytes, and cytokine serum levels were analyzed in mice fed the different carrots for 30 days. Principal component analysis (PCA) was performed in mice fed the Danish carrots. The consumption of the 'more organic' O2 and O3 carrots induced some changes in lymphocyte populations, including an increase in regulatory T cells. In Italian carrots more differences between ORG and CV were observed in the first as compared to the second year. No relevant differences were observed in cytokine secretion. PCA showed a clear separation among mice fed the O1, O2, O3 and D-CV carrots.

CONCLUSIONS: Although a great variability was observed between the two years, an immune stimulation was found after the ORG carrot consumption.

Keywords: organic carrots; immunity; intraepithelial lymphocytes; lamina propria lymphocytes; regulatory T cells

INTRODUCTION

The demand for organic (ORG) products is continuously growing, since consumers perceive them as healthier, safer and of higher nutrient content than conventional (CV) food.[1] Limited research has been performed up to now in studying the real impact of ORG food on health. Most studies deal with differences in nutrient content of ORG *versus* CV products; however, the outcome on improved nutritional quality of ORG vegetables is not fully consistent.[2–5] In addition, results from such studies can only speculatively be connected to health effects. Thus the scientific evidence to recommend ORG over CV vegetables in public health terms is still insufficient.[1,6–8]

Carrots are one of the most popular vegetables worldwide, and the fresh carrot market has been increasing over the past few decades. Carrots are considered one of the healthiest vegetables for their high phytochemicals content, mainly carotenoids and polyacetylenes, including falcarinol, which may exert immunomodulatory and anti-inflammatory activity.[9–12] A higher carotenoid content has been found in ORG as compared to CV carrots,[13,14] although other studies have reported opposite results or did not find differences.[15,16] A possible cause for such discrepancy has been ascribed to the variability in the farming system, harvest year, climate, soil conditions and degree of ripeness. A recent study conducted on men fed ORG and CV carrots concluded that the agricultural system had no effect on carotenoid content and bioavailability, either on the antioxidant status or immune parameters.[17] Moreover, in another study no differences were found in polyacetylene content in ORG and CV carrot growth systems, while significant changes were observed when comparing two consecutive harvest years.[18] Similarly, some authors found that year, but not cultivation strategy, induced variations in protein utilization and energy value of several vegetables, including carrots, in a rat model.[19]

Studies on the health effects of ORG carrots are lacking and still controversial; thus there is still a need to deepen insight into the health implications, as well as to investigate whether the potential benefits may be related to the ORG growing conditions, namely fertilization strategy, previous crop and weed management strategy, rather than to other factors, such as production year, seasonal variations, environmental conditions from one farm to the next, and length of time the soil has been

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organically handled, as several years can be necessary to obtain a soil of good 'organic' quality.[1]

On the basis of the above considerations, the aim of the present work was to provide novel information on the impact of ORG carrots on health, by considering intestinal and systemic immunity and by comparing two production years and three varieties grown in two different geographical locations. For such purposes, we analyzed the leading actors of gut-associated lymphoid tissue (GALT), namely intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs). IELs and LPLs form highly specialized lymphoid compartments, and play a critical role in the mucosal immune system regulation by providing immune surveillance for the epithelium. Indeed, the intestinal mucosa represents the first line of defense against pathogens and dangerous environmental agents, and ensures a relevant immune defense. IELs, which are interspersed with enterocytes, are the first cells encountering luminal antigens, as they establish the closest contact with the lumen, whereas LPLs, which reside in the underlying mucosa, act later.[20–22] Different T and B lymphocyte subsets were analyzed in this study, including the regulatory $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (Treg), which are fundamental for GALT homeostasis. These cells are pivotal players in the maintenance of immune tolerance to self and environmental antigens present in ingested foods, and are specialized in avoiding hypersensitivity reactions and suppressing excessive or misguided immune responses that can be harmful to the host.[23,24]

EXPERIMENTAL

Carrot samples

Carrots were cultivated in 2-year field trials in two different geographical locations: Denmark (Research Centre Aarslev, Funen) and Italy (Fiumicino, Rome). The Danish carrots (*Daucus carota* L., *Bolero* variety) were grown in a Typic Agrudalf soil, with the 0–0.5 m soil layer containing 11 g kg^{-1} C, 170 g kg^{-1} clay, 280 g kg^{-1} silt and 530 g kg^{-1} sand; the 0.5–1 m layer contained 2 g kg^{-1} C, 210 g kg^{-1} clay, 260 g kg^{-1} silt and 530 g kg^{-1} sand. The pH was 6.7 and the content of phosphorus and potassium was 27 and 135 mg kg^{-1}, respectively, in the 0–0.25 m soil layer. The carrots were grown in 2007 (first year) and 2008 (second year) in four different cropping systems for vegetable production: one CV (D-CV) and three ORG (O1, O2 and O3). The three ORG crop rotations had increasing content of fertility-building crops (undersown legume green manures and autumn catch crops) for nutrient management and intercrops to increase biodiversity and natural mechanisms for pest regulation. In particular, the O1 system was similar to the D-CV one, being without fertility-building crops in the crop rotation, and using organic rather than inorganic fertilizers, and no synthetic pesticides. In the O2 system, fertility-building crops had been grown as undersown green manures or autumn catch crops after harvest of the main crop as often as possible. In the O3 system, strips of the undersown green manure from one autumn had been left to grow as intercrops between the vegetable crop rows in the following seasons. Details are shown in Table 1 and further details on the cropping systems, carrot yields and harvest quality are given in a recent publication by Thorup-Kristensen *et al.*[25] The Italian field trials had been performed in 2008 (first year) and 2009 (second year) with two different carrot varieties (*Maestro* and *Excelso*), and were laid out at two farms, one CV and one ORG, situated close to each other. The two varieties were grown in both farms in both years on plots of about 1 ha each. The area was flat and soils were extremely sandy. The main difference of the soil condition between the two farms was a slightly higher organic matter content in the ORG field, which induces in extremely sandy soils highly different performances in terms of water and nutrient availability to the crops. Concerning the management system, crop rotation (*versus* monoculture in CV) and green manure were used in the ORG farm. The carrots were harvested at the same time from all systems at each site in both years and quickly delivered to laboratories as coded samples, and were not decoded until finishing results collection each year. Carrots were homogenized, freeze-dried and stored at −80 °C until diet preparation.

Animals and diets

Balb/c 21-day-old mice, obtained from Charles River Laboratories (Como, Italy), were kept at 23 °C with a 12 h light–dark cycle and fed *ad libitum* for 30 days with diets containing each of the ORG or CV carrots (70 g freeze-dried carrots kg^{-1} diet). A 200 g kg^{-1} casein balanced diet, without carrots, was also used as control (C) diet. The Danish carrot-containing diets were named O1, O2, O3 and D-CV diets, whereas the Italian carrot-containing diets were named ORG *Maestro*, CV *Maestro*, ORG *Excelso* and CV *Excelso* diets. The diet compositions are shown in Table 2. Body weight and food intake were recorded every week and every other day, respectively. At the end of the experimental periods, animals were anaesthetized with intraperitoneal injection of pentobarbital (10 mg kg^{-1}), blood was drawn via cardiac puncture, and small intestine and spleen were excised and placed in cold phosphate-buffered saline (PBS). Animal studies were performed under conditions approved by the National Health Ministry, Department of Food, Nutrition and Animal Health.

Lymphocyte preparation

Lymphocytes were prepared from different districts: splenocytes from spleen, peripheral blood mononuclear cells from blood, and LPLs and IELs from small intestine.

Splenocytes

Spleens were smashed with a 1 mL plastic syringe piston, and the released lymphocytes were washed with PBS, separated on Ficoll gradient (Ficoll plaque-plus, GE Healthcare, Milan, Italy), and resuspended in PBS.

IELs

Colons were placed on ice in 10 mL RPMI-1640 medium (Sigma, Milan, Italy), washed twice with cold PBS, longitudinally opened and cut into small-size pieces. Intestinal pieces were washed in Hank's balanced salt solution Ca^{2+} and Mg^{2+} free (HBSS-CMF) and stirred twice for 45 min at 37 °C in an orbital shaker in HBSS-CMF added with 100 g L^{-1} fetal calf serum (FCS; Euroclone, Milan, Italy), 1×10^5 U L^{-1} penicillin, 100 mg L^{-1} streptomycin, 1 mmol L^{-1} ethylenediaminetetraacetic acid, 5 mmol L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), and 1 mmol L^{-1} dithiotreithol. The solution was passed through 100 and 40 µm nylon cell strainers (BD Falcon, Milan, Italy) and centrifuged at 650 × *g*. IELs were isolated from enterocytes by discontinuous 440/670 g L^{-1} Percoll gradient (Percoll™, GE Healthcare) in RPMI-1640 medium, and centrifuged at 650 × *g* for 25 min.

Table 1. Description of the four cropping systems in the Danish field experiment where carrots were harvested in 2007 and 2008

	D-CV Conventional	O1 Organic	O2 Organic	O3 Organic
Description	High external inputs No fertility-building crops	High external inputs No fertility building crops	Low external inputs Catch crops, green manure	Low external inputs Catch crops, green manure, living mulch
Pest management	Pesticides	Mechanical weeding	Mechanical weeding	Mechanical weeding Living mulch
Crop rotation				
Soil cover winter prior to oat (*Avena sativa*)	Bare soil	Bare soil	Catch crop (*Raphanus sativus*)	Catch crop (*R. sativus*)
Fertilization of oat	90 kg ha^{-1} N as NPK fertilizer	47 kg ha^{-1} N as pig slurry	0 kg ha^{-1} N	0 kg ha^{-1} N
Soil cover winter between oat and carrots	Bare soil	Bare soil	Undersown green manure (*Anthyllis vulneraria, Medicago lupulina, Trifolium repens, Lolium perenne*)	Undersown green manure (*Sanguisorba minor, Lotus corniculatus*)
Fertilization of carrots	120 kg ha^{-1} N as NPK fertilizer	56 kg ha^{-1} N as pig slurry	0 kg ha^{-1} N	0 kg ha^{-1} N

Table 2. Diet compositions

Component	Carrot diet (g kg^{-1})	Control diet (g kg^{-1})
Casein	192	200
Carrots[a]	70	–
Starch	471	480
Sucrose	117	180
Soya oil	100	100
Saline mix	35	35
Vitamin mix	10	10
Choline chloride	2	2
Methionine	3	3

[a] Homogenized freeze-dried, coming from Danish and Italian fields.

LPLs

After separation from IELs, colon fractions were washed with HBSS and stirred for 1 h at 37 °C with 10 mL RPMI-1640 medium containing 100 g L^{-1} FCS, 5 mmol L^{-1} Hepes, 1.2 × 10^5 U L^{-1} collagenase I and 10 mg L^{-1} DNAse (both from Sigma). Cells were filtered and centrifuged as described for IELs.

Flow cytometry analysis

The following monoclonal antibodies were used for lymphocyte surface staining: fluorescein isothiocyanate (FITC) anti-CD3 (clone 17.12), phycoeritrin (PE) anti-CD4 (clone GK1.5), phycoeritrin–cyanine 5 (PE-Cy5) anti-CD8 (clone 53–67), PE anti-CD19 (clone ID3), peridinin–chlorophyll–protein (PerCP) anti-CD45 (clone 30-F11), and anti-CD16/CD32 (clone 2.4G2) (BD Pharmingen, Milan, Italy). Each antibody was titrated to determine the optimal concentration for maximal staining. The IELs and LPLs (1 × 10^6 cells) were pre-incubated for 20 min with anti-CD16/CD32 to block Fc receptors, thus avoiding non-specific binding. Cells were then washed and labeled with an appropriate mixture of antibodies or isotype-matched controls for 30 min, centrifuged at 650 × *g* and resuspended in FacsFlow (BD Biosciences, Milan, Italy). Flow cytometry analysis was performed using a FACSCalibur instrument (BD Biosciences). To exclude dead/dying cells and therefore non-specific antibody-binding cells, lymphocytes were gated according to forward and side scatter. The percentage of B lymphocytes was calculated on leukocyte gate (CD45$^+$), whereas the CD4$^+$ and CD8$^+$ subsets were calculated on CD3$^+$ gate. At least 10 000 events were acquired. Data were analyzed using CellQuestPro software (BD Biosciences).

The analysis of CD4$^+$ CD25$^+$ Foxp3$^+$ cells was performed in IELs, LPLs and spleen with a specific Kit (eBioscience, San Diego, CA, USA) staining CD4 (FITC), CD25 (PE) and transcription factor Foxp3 (PE-Cy5), according to the manufacturer's instructions. Briefly, lymphocytes were first stained for surface markers CD4 and CD25, for 30 min at 4 °C, then permeabilized and stained for intracellular Foxp3, for 30 min at 4 °C. Cells were analyzed by flow cytometry and the percentage of CD25$^+$ Foxp3$^+$ cells was calculated on CD4$^+$ gate.

Cytokine analysis

Levels of serum cytokines were analyzed in mice fed the Danish and Italian diets from the first year of carrot harvest using the mouse inflammatory cytometric bead array (CBA, BD Biosciences) for interleukin (IL)-12-p70, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1 and IL-10 detection, according to the manufacturer's specifications. Briefly, microbeads with distinct fluorescence intensities, coated with capture antibody specific for each cytokine, were incubated with serum samples and PE-conjugated detection antibodies for 2 h. The samples were then washed, resuspended in 300 µL buffer and analyzed by flow cytometry, using FCAP analysis software (BD Biosciences). The theoretical detection limit for each cytokine is defined by the kit manufacturer as the corresponding concentration at two standard deviations above the median fluorescence of 20 replicates of the negative control (0 pg mL^{-1}). Detection limits were for IL-12-p70: 10.7 pg mL^{-1}; for IFN-γ: 2.5 pg mL^{-1}; for TNF-α: 7.3 pg mL^{-1}; for MCP-1: 52.7 pg mL^{-1}; for IL-10: 17.5 pg mL^{-1}.

Statistical univariate analysis

At least eight mice for each group of diets were used. The significance of differences was evaluated by one-way analysis

Table 3. Body weight and food consumption of mice fed the Danish (*Bolero* variety) and Italian (*Maestro* and *Excelso* varieties) carrot diets (70 g kg^{-1}) and control diet (C, casein 200 g kg^{-1})

	Body weight (*g*)		Food intake (g day^{-1})
	Initial	Final	
First year			
Bolero			
O1	24.1 ± 0.7	29.0 ± 1.4	4.3 ± 0.3
O2	24.0 ± 1.2	26.9 ± 2.6	4.0 ± 0.4
O3	23.5 ± 0.9	26.4 ± 1.3	4.1 ± 0.2
D-CV	23.9 ± 1.1	27.6 ± 1.6	4.2 ± 0.4
Maestro			
ORG	22.4 ± 0.9	27.1 ± 1.8	4.2 ± 0.3
CV	24.9 ± 1.3	27.3 ± 2.0	4.0 ± 0.3
Excelso			
ORG	23.6 ± 1.3	26.9 ± 1.6	3.8 ± 0.2
CV	24.0 ± 0.9	27.4 ± 1.4	3.8 ± 0.2
C	23.0 ± 0.1	26.8 ± 1.3	3.7 ± 0.4
Second year			
Bolero			
O1	23.6 ± 1.6	29.5 ± 3.0	4.1 ± 0.3
O2	24.6 ± 0.7	26.1 ± 0.8	4.4 ± 0.3
O3	24.0 ± 2.0	26.1 ± 2.8	4.0 ± 0.3
D-CV	22.9 ± 1.5	27.9 ± 3.1	4.0 ± 0.3
Maestro			
ORG	21.1 ± 0.8	25.3 ± 2.1	4.1 ± 0.5
CV	21.6 ± 2.1	26.4 ± 2.9	4.0 ± 0.3
Excelso			
ORG	23.3 ± 1.7	27.3 ± 1.5	3.7 ± 0.2
CV	22.0 ± 1.2	27.4 ± 3.1	3.5 ± 0.5
C	24.1 ± 1.0	27.6 ± 2.3	3.9 ± 0.4

O1, O2, O3: organic, D-CV: conventional; ORG: organic, CV: conventional. Data represent means ± SD of at least eight mice per group.

of variance (ANOVA), followed by Fisher's test. Differences with *P*-values <0.05 were considered significant. Statistical analysis was performed with Statistica for Windows software package' (Stat Soft Inc., Tulsa, OK, USA).

Principal component analysis (PCA)

The evaluation of possible systemic effects of the different diets was assessed by applying statistical multivariate analysis on spleen, IELs and LPLs subpopulations analyzed by flow cytometry, from mice fed the O1, O2, O3 and D-CV diets, first harvest year (in which more differences were observed with univariate analysis), and the C diet. For each sample, cell percentages were normalized for the number of acquired gated events, and these data were collected in a matrix having 40 rows (eight animals for each diet) and 12 columns ($CD4^+$, $CD8^+$, $CD19^+$ and $CD4^+$ $CD25^+$ $Foxp3^+$ from each of the three compartments). PCA was performed on the data matrix with Unscrambler vers. 9.8 software (Camo Software AS, Oslo, Norway). Data were mean centered and standardized, in order to equalize the importance of the variation of each variable independently on its numerical size. Full cross-validation (leave-one-out) was performed to assess the robustness of the PCA analysis and detect the presence of possible outliers: two samples were detected – actually two mice fed the C and O2 diets respectively – and were excluded from further analysis.

RESULTS

Body weight and food consumption

After consumption of the Danish carrots, the body weight of mice fed the O1, O2 and O3 diets did not differ from that of mice fed the D-CV diet. The production year had no effect on body weight. Similarly, there was no difference in the final body weight after 30 days' consumption of the Italian ORG and CV diets, independently of the production year. These results indicate that ORG and CV carrots did not impair mice growth (Table 3). The food intake was similar in all mice fed the different diets (Table 3).

Effect of ORG and CV carrots on lymphocyte populations

To assess the impact of ORG and CV carrot consumption on the local and systemic immunity, the phenotypic analysis of lymphocytes isolated from the small intestine, spleen and blood was performed. In general, some changes on lymphocyte subsets were observed after ORG and CV carrot consumption, which, however, were different after consumption of carrots of the first and second cultivation years. Relating to the *Bolero* variety, the O2 and O3 diets, containing carrots of both the first and second cultivation years, had similar effects on the lymphocyte population distribution in intestine, spleen and blood, and differed from that of the O1 diet. Concerning the first-year carrot production and comparing to the D-CV diet, in IELs the consumption of the O2 and O3 diets resulted in lower $CD4^+$ and higher $CD8^+$ cells, and the consumption of the O2 diet induced a higher $CD19^+$ percentage (Table 4). In addition, the $CD4^+$ and $CD8^+$ percentages after the O2 and O3 diets were similar to those of the C diet (Table 5). After the second-year carrot consumption, the lymphocyte populations of IELs did not differ from those observed after the first year carrot consumption, with the exception of $CD8^+$ cell distribution, which was similar in mice fed the O1, O2, O3 and D-CV diets (Table 4). In LPLs, higher percentages of $CD8^+$ and $CD4^+$ $CD25^+$ $Foxp3^+$ cells were observed after the O2 and O3 than D-CV diets of the first year. After the second year carrot consumption, $CD8^+$ remained higher, and the O2 diet induced a higher percentage of $CD19^+$ and a small but not significant increase of $CD4^+$ $CD25^+$ $Foxp3^+$ cells. In the spleen, a higher percentage of $CD4^+$ and $CD4^+$ $CD25^+$ $Foxp3^+$ cells and a lower percentage of $CD8^+$ were observed after the O2 and O3 than the D-CV diets of the first year. These changes were not maintained after the second-year carrot consumption: $CD4^+$ and $CD4^+$ $CD25^+$ $Foxp3^+$ cell percentages were similar in all mice (Table 4); $CD8^+$ increased after the O2 and O3 diets (Table 4); $CD8^+$ and $CD4^+$ $CD25^+$ $Foxp3^+$ cells after the O2 and O3 diets were similar to those observed after the C diet (Table 5). In the blood, only an increase of $CD19^+$ was found after the O2 and O3 as compared to the D-CV and C diets of the first year, which, however, was not seen after the second-year carrot consumption (Table 4). Relating to the O1 diet, the main changes after first-year carrot consumption were an increase of $CD4^+$ and $CD19^+$ cells in IELs, as well as an increase of $CD4^+$ and $CD4^+$ $CD25^+$ $Foxp3^+$ cells in LPLs, as compared to the D-CV and C diets. These changes were not maintained after the second-year carrot consumption, with the exception of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in the LPLs (Table 4).

Concerning the Italian carrots, more changes were observed after the first than second year carrot consumption. For *Maestro* variety, the ORG diet of the first year induced higher $CD8^+$ subpopulation in IELs and LPLs, and higher $CD19^+$ cells in LPLs, as compared to the CV diet. In addition, $CD4^+$, $CD8^+$ and $CD19^+$ subpopulations increased in the spleen, whereas $CD19^+$ cells decreased in the blood. After the second-year carrot consumption,

Table 4. Lymphocyte population percentages of IELs, LPLs, blood and spleen of mice fed the Danish (*Bolero* variety) organic (O1, O2, O3) and conventional (D-CV) carrot diets

	First year				Second year			
Bolero	O1	O2	O3	D-CV	O1	O2	O3	D-CV
IELs								
$CD4^+$	$19.3^{**} \pm 4.2$	$10.5^{*} \pm 1.6$	$8.2^{*} \pm 2.1$	13.4 ± 2.7	14.0 ± 4.0	$9.1^{*} \pm 0.6$	$7.2^{*} \pm 0.8$	11.1 ± 0.9
$CD8^+$	60.7 ± 9.9	$81.2^{*} \pm 2.2$	$85.8^{*} \pm 2.7$	63.4 ± 10.4	81.1 ± 3.0	83.4 ± 3.6	82.8 ± 3.8	81.4 ± 2.4
$CD19^+$	$26.8^{**} \pm 5.4$	$13.1^{\S} \pm 5.6$	9.6 ± 4.6	7.9 ± 0.4	$4.5^{\#} \pm 1.8$	$8.0^{\S\S} \pm 4.2$	4.9 ± 0.9	5.8 ± 3.3
$CD4^+$ $CD25^+$ $Foxp3^+$	3.9 ± 1.0	4.3 ± 1.5	3.4 ± 0.8	3.3 ± 0.9	3.8 ± 1.9	3.7 ± 1.3	4.3 ± 1.5	3.4 ± 1.6
LPLs								
$CD4^+$	$62.6^{**} \pm 3.2$	42.0 ± 4.3	48.8 ± 4.6	42.3 ± 7.9	$62.6^{**} \pm 8.2$	$52.0^{\#} \pm 2.4$	49.0 ± 17.2	$59.3^{\#} \pm 11.2$
$CD8^+$	25.0 ± 5.5	$52.3^{*} \pm 3.2$	$42.5^{*} \pm 3.4$	22.6 ± 2.5	26.6 ± 6.1	$37.6^{*\#} \pm 1.9$	$31.4^{*\#} \pm 3.9$	23.9 ± 2.9
$CD19^+$	16.4 ± 0.8	15.1 ± 1.9	16.2 ± 5.5	20.5 ± 5.4	$26.9^{\S\S\#} \pm 3.2$	$27.3^{\S\S\#} \pm 4.9$	13.1 ± 4.2	17.9 ± 4.9
$CD4^+$ $CD25^+$ $Foxp3^+$	$12.8^{\S\S} \pm 0.5$	$7.7^{\S\S} \pm 2.8$	$7.4^{\S\S} \pm 2.2$	4.9 ± 0.9	$10.1^{**} \pm 4.7$	7.0 ± 1.2	5.5 ± 1.1	5.6 ± 1.1
Blood								
$CD4^+$	77.3 ± 2.5	79.0 ± 1.4	78.7 ± 1.5	76.1 ± 1.9	76.6 ± 1.4	76.5 ± 3.4	74.6 ± 3.7	75.5 ± 2.9
$CD8^+$	19.7 ± 2.3	19.8 ± 1.8	20.2 ± 1.5	20.4 ± 2.2	21.0 ± 1.5	20.0 ± 2.4	21.5 ± 2.8	21.8 ± 3.0
$CD19^+$	17.1 ± 4.2	$26.2^{*} \pm 8.2$	$31.9^{*} \pm 5.6$	16.3 ± 5.8	22.2 ± 7.8	26.0 ± 10.2	25.8 ± 13.0	29.7 ± 19.0
Spleen								
$CD4^+$	57.1 ± 6.2	$73.3^{*} \pm 2.9$	$72.8^{*} \pm 4.0$	58.7 ± 3.8	59.7 ± 2.8	$57.8^{\#} \pm 2.6$	$55.3^{\#} \pm 5.2$	50.4 ± 14.5
$CD8^+$	34.0 ± 7.4	$23.0^{*} \pm 3.6$	$24.0^{*} \pm 4.0$	33.8 ± 2.2	$26.8^{\#} \pm 3.7$	$33.5^{*\#} \pm 2.8$	$36.2^{*\#} \pm 4.2$	26.6 ± 8.9
$CD19^+$	48.6 ± 16.2	47.7 ± 8.2	56.5 ± 4.4	56.6 ± 5.5	54.1 ± 9.9	$61.5^{\#} \pm 4.1$	60.9 ± 3.4	54.3 ± 5.5
$CD4^+$ $CD25^+$ $Foxp3^+$	11.4 ± 1.1	$14.1^{*} \pm 1.0$	$15.1^{*} \pm 0.9$	9.0 ± 1.3	11.2 ± 2.0	12.6 ± 1.3	13.0 ± 1.3	11.0 ± 1.5

Data represent means ± SD of at least eight mice per group.
IELs, first year. $CD4^+$ and $CD19^+$: ** $P < 0.01$ O1 vs. all; $CD4^+$ and $CD8^+$: * $P < 0.05$ O2 and O3 vs. O1 and D-CV; $CD19^+$: § $P < 0.05$ O2 vs. D-CV. Second year. $CD4^+$: * $P < 0.05$ O2 and O3 vs. O1 and D-CV; $CD19^+$: §§ $P < 0.05$ O2 vs. O1 and O3.
LPLs, first year. $CD8^+$: * $P < 0.05$ O2 and O3 vs. O1 and D-CV; $CD4^+$ $CD25^+$ $Foxp3^+$: §§ $P < 0.05$ O1, O2 and O3 vs. D-CV. Second year. $CD4^+$: ** $P < 0.05$ O1 vs. O2 and O3; $CD8^+$: * $P < 0.05$ O2 and O3 vs. O1 and D-CV; $CD19^+$: §§ $P < 0.05$ O1 and O2 vs. O3; $CD4^+$ $CD25^+$ $Foxp3^+$: ** $P < 0.05$ O1 vs. all.
Blood. * $P < 0.05$ O2 and O3 vs. O1 and D-CV.
Spleen, first and second years. $CD4^+$, $CD8^+$ and $CD4^+$ $CD25^+$ $Foxp3^+$: * $P < 0.05$ O2 and O3 vs. O1 and D-CV. All: $^{\#}$ $P < 0.05$ second year vs. first year, same treatment.

Table 5. Lymphocyte population percentages of IELs, LPLs, blood and spleen of mice fed the control (C) diet

	First year	Second year
IELs		
$CD4^+$	10.7 ± 1.2	11.4 ± 2.2
$CD8^+$	80.5 ± 3.7	77.1 ± 4.7
$CD19^+$	6.8 ± 0.9	4.6 ± 3.6
$CD4^+$ $CD25^+$ $Foxp3^+$	4.6 ± 1.5	$7.2^{\#} \pm 1.5$
LPLs		
$CD4^+$	40.9 ± 6.1	44.9 ± 4.1
$CD8^+$	24.9 ± 5.9	28.2 ± 4.2
$CD19^+$	15.3 ± 3.9	17.5 ± 2.7
$CD4^+$ $CD25^+$ $Foxp3^+$	8.6 ± 2.7	6.1 ± 0.9
Blood		
$CD4^+$	79.1 ± 2.5	78.2 ± 2.7
$CD8^+$	18.7 ± 1.9	19.9 ± 2.8
$CD19^+$	14.9 ± 2.9	$20.6^{\#} \pm 5.7$
Spleen		
$CD4^+$	68.4 ± 4.2	$61.0^{\#} \pm 3.7$
$CD8^+$	28.9 ± 3.9	32.8 ± 3.2
$CD19^+$	47.4 ± 3.5	52.1 ± 4.3
$CD4^+$ $CD25^+$ $Foxp3^+$	11.1 ± 1.3	12.6 ± 0.7

Data represent means ± SD of at least eight mice per group.
$^{\#}$ $P < 0.05$ second year vs. first year.

an opposite trend of $CD19^+$ cells was found in the blood. In addition, in IELs $CD4^+$ cells were lower and $CD8^+$ cells higher after the ORG than CV diet (Table 6). For *Excelso* variety, the ORG diet of the first year induced higher $CD8^+$ cells in IELs and LPLs, and lower $CD19^+$ and $CD8^+$ cells in the spleen, as compared to the CV diet. In the blood, $CD19^+$ cells increased. After the second-year ORG diet, only a lower $CD4^+$ percentage in IELs was observed, as compared to the CV diet (Table 7).

PCA

The first three components yielded by PCA accounted for 72.98% of the overall variance, with individual values of 35.85%, 21.11% and 16.02% for PC1, PC2 and PC3, respectively. The three-dimensional plot of the scores (individual values of the PCs for each sample, Fig. 1(A)) shows a clear separation among the mice fed the O1, O2, O3, D-CV or C diets. The two-dimensional projections along PC1/PC2 (Fig. 1(B)) and PC1/PC3 (Fig. 1(C)) planes, allowing a clearer representation of the analysis, show that: PC1 is mainly responsible for the separation of the mice fed the C, D-CV and O1 diets from mice fed the O2 and O3 diets; PC2 discriminates mice fed the C diet from the remaining mice, and mice fed the D-CV diet from mice fed the O1, O2 and O3 diets; and PC3 allows the separation between mice fed the O2 and O3 diets. The lymphocyte subpopulations mainly responsible for these discrimination are identified by inspection of the loadings (Table 8, correlation coefficients of the original variables with the PCs): PC1 shows strong significant direct correlation with spleen $CD8^+$ cells

Table 6. Lymphocyte population percentages of IELs, LPLs blood and spleen of mice fed the Italian (*Maestro* variety) organic (ORG) and conventional (CV) carrot diets

	First year		Second year	
Maestro	ORG	CV	ORG	CV
IELs				
$CD4^+$	10.6 ± 2.6	12.9 ± 2.5	$6.0^* \pm 3.3$	10.4 ± 1.8
$CD8^+$	$87.6^* \pm 1.3$	78.8 ± 6.8	$85.6^* \pm 3.9$	69.8 ± 6.7
$CD19^+$	5.1 ± 2.1	6.4 ± 3.2	5.0 ± 2.9	5.6 ± 2.4
$CD4^+$ $CD25^+$ $Foxp3^+$	3.3 ± 0.9	3.6 ± 1.1	3.6 ± 1.6	2.7 ± 0.8
LPLs				
$CD4^+$	66.4 ± 4.1	64.4 ± 6.4	$55.3^\# \pm 9.5$	$54.3^\# \pm 10.3$
$CD8^+$	$25.5^* \pm 4.1$	17.9 ± 4.8	$32.9^\# \pm 5.2$	$37.4^\# \pm 10.5$
$CD19^+$	$25.4^* \pm 4.8$	19.8 ± 1.9	24.5 ± 8.1	24.1 ± 8.8
$CD4^+$ $CD25^+$ $Foxp3^+$	9.2 ± 1.3	8.2 ± 2.3	$6.2^\# \pm 0.8$	$5.9^\# \pm 2.8$
Blood				
$CD4^+$	78.4 ± 1.7	76.1 ± 3.3	77.5 ± 2.1	78.8 ± 2.8
$CD8^+$	20.0 ± 1.3	21.7 ± 2.6	19.7 ± 2.6	18.9 ± 2.0
$CD19^+$	$14.1^* \pm 2.4$	20.8 ± 6.4	$21.2^{*\#} \pm 6.6$	$14.3^\# \pm 3.9$
Spleen				
$CD4^+$	$61.2^* \pm 2.7$	52.0 ± 4.3	60.1 ± 4.6	$60.6^\# \pm 6.8$
$CD8^+$	$34.6^* \pm 2.7$	30.6 ± 1.4	34.1 ± 2.4	34.0 ± 5.9
$CD19^+$	$65.3^* \pm 3.4$	57.9 ± 1.8	59.2 ± 5.2	63.3 ± 5.8
$CD4^+$ $CD25^+$ $Foxp3^+$	9.4 ± 2.0	10.1 ± 1.7	10.4 ± 1.6	10.3 ± 1.5

Data represent means $\pm$ SD of at least eight mice per group.
$^*P < 0.05$ ORG vs. CV, same year; $^\#P < 0.05$ second year vs. first year, same treatment.

Table 7. Lymphocyte population percentages of IELs, LPLs, blood and spleen of mice fed the Italian (*Excelso* variety) organic (ORG) and conventional (CV) carrot diets

	First year		Second year	
Excelso	ORG	CV	ORG	CV
IELs				
$CD4^+$	4.2 ± 0.9	3.9 ± 1.0	$5.5^* \pm 1.1$	$11.3^\# \pm 4.4$
$CD8^+$	$74.2^* \pm 7.6$	82.7 ± 3.7	73.8 ± 11.8	$69.2^\# \pm 7.9$
$CD19^+$	4.7 ± 2.4	5.2 ± 1.6	6.9 ± 2.6	$10.8^\# \pm 6.8$
$CD4^+$ $CD25^+$ $Foxp3^+$	3.0 ± 1.6	2.7 ± 1.2	4.7 ± 0.7	$5.5^\# \pm 1.4$
LPLs				
$CD4^+$	50.5 ± 4.4	45.9 ± 6.6	58.9 ± 7.0	45.0 ± 5.3
$CD8^+$	$32.1^* \pm 6.3$	37.7 ± 2.4	$25.8^\# \pm 4.2$	$22.6^\# \pm 6.4$
$CD19^+$	19.7 ± 8.2	20.4 ± 8.5	20.2 ± 10.6	19.2 ± 6.0
$CD4^+$ $CD25^+$ $Foxp3^+$	6.8 ± 2.0	8.5 ± 4.1	$8.1^\# \pm 2.4$	$5.5^\# \pm 1.4$
Blood				
$CD4^+$	74.5 ± 3.4	76.2 ± 1.9	74.0 ± 2.6	77.4 ± 3.2
$CD8^+$	21.1 ± 1.9	19.7 ± 1.2	21.8 ± 1.8	19.4 ± 3.2
$CD19^+$	$22.4^* \pm 7.8$	14.1 ± 6.2	27.5 ± 8.5	$21.8^\# \pm 7.4$
Spleen				
$CD4^+$	65.7 ± 2.3	64.4 ± 2.5	$57.9^\# \pm 5.7$	60.5 ± 4.3
$CD8^+$	$23.1^* \pm 2.0$	26.7 ± 3.3	$31.9^\# \pm 2.8$	$33.5^\# \pm 3.0$
$CD19^+$	$59.0^* \pm 4.7$	63.5 ± 2.0	57.6 ± 6.9	$54.1^\# \pm 5.5$
$CD4^+$ $CD25^+$ $Foxp3^+$	13.0 ± 1.1	12.3 ± 1.0	12.6 ± 0.5	10.9 ± 1.8

Data represent the means $\pm$ SD of at least eight mice per group. $^*P < 0.05$ ORG vs. CV, same year; $^\#P < 0.05$ second year vs. first year, same treatment.

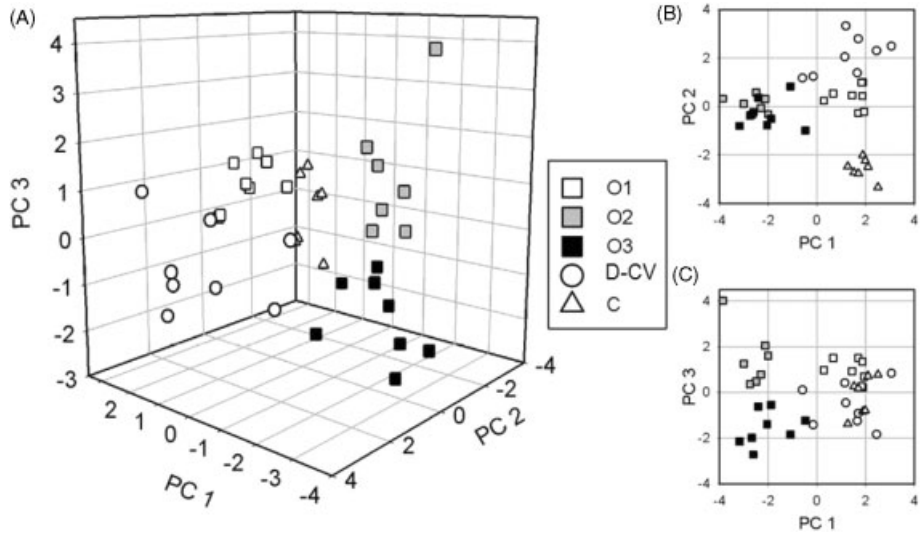

Figure 1. PCA scores plot of the first three principal components from mice fed the Danish (*Bolero* variety) organic (O1, O2, O3) and conventional (D-CV) carrot diets (first harvest year), and the control (C) diet: three-dimensional view (A), projection along the PC1, PC2 plane (B), and projection along the PC1, PC3 plane (C).

Table 8. Loadings of the first three principal components from mice fed the Danish (*Bolero* variety) organic (O1, O2, O3) and conventional (D-CV) carrot diets (first harvest year), and the control (C) diet

	PC 1	PC 2	PC 3
IELs			
$CD4^+$	**−0.730**	−0.069	0.359
$CD8^+$	**−0.874**	−0.178	0.016
$CD19^+$	−0.611	0.333	0.491
$CD4^+$ $CD25^+$ $Foxp3^+$	−0.402	−0.105	0.556
LPLs			
$CD4^+$	0.277	**0.809**	−0.158
$CD8^+$	**−0.708**	0.488	0.149
$CD19^+$	−0.568	−0.069	−0.478
$CD4^+$ $CD25^+$ $Foxp3^+$	0.599	**−0.684**	0.045
Spleen			
$CD4^+$	0.368	−0.278	**0.730**
$CD8^+$	**0.874**	0.071	0.178
$CD19^+$	−0.419	−0.402	**−0.625**
$CD4^+$ $CD25^+$ $Foxp3^+$	−0.366	**−0.876**	0.099

Significance level is associated with loadings (Pearson's correlation coefficients) >0.325 in absolute value (36 degrees of freedom). Positive signs indicate a direct correlation, whereas negative signs indicate an inverse correlation with the PC. Most significant correlations are indicated in bold.

and a significant inverse correlation with LPL and IEL $CD8^+$ cells; PC2 is mainly ruled by direct correlation with LPL $CD4^+$ cells and inverse correlation with LPL $CD4^+$ $CD25^+$ $Foxp3^+$ cells; and PC3 has a main significant direct correlation with spleen $CD4^+$ cells counterbalanced by an inverse correlation with spleen $CD19^+$ cells.

Cytokine analysis

In order to assess whether ORG and CV diets, from Danish and Italian field trials, first harvest year, could influence the cytokine profile, pro- and anti-inflammatory cytokine levels were measured in serum. The levels of all the analyzed cytokines, namely IL-12-p70, IL-6, IFN-γ, TNF-α, MCP-1 and IL-10, were very low, and no significant differences between ORG and CV of the three varieties were found, except for a small increase of IL-6 and MCP-1 serum secretion induced by the O1 diet (Table 9).

DISCUSSION

Works on health benefits of ORG foods are scarce up to now, the majority of them looking at antioxidant activity,[17,26] whereas immunological studies are extremely rare, and exclusively related to systemic immune response.[27,28] To our knowledge, this is the first study on the impact of ORG food on intestinal immunity. Important aspects for the evaluation of the health effects of ORG food consumption are also considered in this work, namely the influence of environmental and growing conditions on the ORG carrot-mediated effect on mice immune response. In fact, we analyzed the impact on intestinal and peripheral immune system of ORG carrots, derived from Danish and Italian field trials from two consecutive harvests years. In particular, we compared ORG with CV of the same variety, *Bolero, Maestro* and *Excelso*. Within the *Bolero* variety, three different ORG cropping systems, ranging from O1, the 'less organic', to O3, the 'most organic', were also compared among them. When comparing ORG with CV foods, several important factors such as geographical location, climate, growth season, and length of time of ORG handling of the soil must be taken into account, to ensure that the eventual differences are systematic and reliable, but often this has not been the case, rendering consistent conclusions quite difficult.[1] For such reason we decided to compare only the farming practice (ORG vs. CV) within each carrot variety, and not the same farming practice among different varieties.

The GALT, the largest immune organ in the body, represents the main response to harmful antigens and microbes, while maintaining tolerance towards food-derived innocuous antigens.[20] The intestinal immune response is mainly triggered by different lymphocyte classes: LPLs and IELs. LPLs constitute the major effector cells along gut mucosal surfaces, whereas IELs are interspersed with enterocytes, thus having direct contact with foreign antigens

Table 9. Cytokine secretion (pg mL^{-1}) in serum of mice fed the Danish (*Bolero* variety) and Italian (*Maestro* and *Excelso* varieties) carrot diets (first harvest year), and the control (C) diet

	IL-6	IL-10	MCP-1	IFN-γ	TNF-α	IL12-p70
Bolero						
O1	60.16* ± 4.6	4.28 ± 1.9	63.82* ± 3.7	1.57 ± 0.2	8.30 ± 1.8	3.54 ± 0.7
O2	3.94 ± 0.3	5.84 ± 0.5	19.77 ± 2.3	1.43 ± 0.2	8.96 ± 2.0	4.05 ± 0.4
O3	3.84 ± 0.5	ND	8.92 ± 0.7	0.51 ± 0.1	5.70 ± 1.2	3.20 ± 0.4
D-CV	2.12 ± 0.1	1.84 ± 0.2	11.24 ± 1.3	0.65 ± 0.1	5.55 ± 1.5	2.51 ± 0.3
Maestro						
ORG	1.82 ± 0.9	ND	10.04 ± 1.7	ND	7.07 ± 0.6	ND
CV	2.63 ± 0.5	2.41 ± 0.2	6.59 ± 0.4	0.58 ± 0.1	2.50 ± 0.3	ND
Excelso						
ORG	0.97 ± 0.9	ND	14.96 ± 1.6	1.17 ± 0.2	2.71 ± 0.3	ND
CV	2.74 ± 0.4	1.09 ± 0.2	11.09 ± 1.0	ND	3.26 ± 0.4	ND
C	8.49 ± 1.0	6.38 ± 0.5	28.98 ± 1.8	2.95 ± 0.1	10.45 ± 0.9	5.60 ± 0.7

O1, O2, O3: organic, D-CV: conventional; ORG: organic, CV: conventional.
Data represent means ± SD of at least eight mice per group. * $P < 0.05$ O1 vs. D-CV.

derived from the gut lumen, and are thought to play a key role in the immune responses toward these antigens.[21,22] The present study reports some significant differences in the main lymphocyte subpopulations considered, indicating an increased stimulation of both local and systemic immune response, and suggesting a positive effect of these two 'more organic' products on the health status. The results show an expansion of Treg cells after ORG carrot consumption. These cells are a subpopulation of $CD4^+$ $CD25^+$ T cells expressing the Foxp3 transcription factor and are crucial for the maintenance of immune homeostasis, in virtue of their cytokine-mediated suppressive activity.[23,29] It is widely accepted that any condition inducing an increase in Treg levels, locally and/or systemically, is considered beneficial.[30–33] Thus the increase of Treg cells suggests a positive effect exerted both locally and systemically by the two 'more organic' carrots of the *Bolero* variety, as compared to the D-CV diet. This is the first evidence of a Treg increase induced by ORG food consumption.

This study highlights that the year effect was stronger than the cultivation system (ORG vs. CV) within the same year, in agreement with previous reports, showing that the weather conditions from year to year changed the outcome of the results.[16,17,19,34]. In the second year climatic conditions in the Italian fields area were very negative, with low temperatures in springtime and high rainfall. This had a general influence on all carrot quality, and might have induced a quality flattening between ORG and CV samples.

Strikingly, the PCA analysis shows a clear separation among the mice fed the O1, O2, O3, D-CV or C diets (first harvest year), indicating that the ORG carrots induce an immune stimulation and strongly influence the immune phenotypes considered in this study. Considering the crucial role of Treg cells in the immune response regulation,[23] the fact that both lamina propria and spleen Treg cells are highly correlated with the same sign to PC2 highlights the importance of Treg cells in determining the discrimination of ORG from D-CV carrots. In addition, the strong correlation of $CD8^+$ subpopulation with PC1, which is inversely correlated with the intestine (both lamina propria and intraepithelial compartments) and directly correlated with the spleen, strengthens the importance of considering both the intestinal and systemic immune response. Interestingly, the recognition of the variables $CD4^+$ and $CD19^+$ in the spleen by the PC3 suggests that both T and B cells are crucial for the group separation.

Cytokines are fundamental soluble immune mediators, whose levels change in response to modifications in the immune balance.[35] The results of serum cytokine secretion in this study indicate that the ORG carrots did not induce an inflammatory status in mice, nor an imbalance between pro- and anti-inflammatory cytokines. These data are particularly relevant considering that possible mycotoxin contaminants affecting the immune system may occur in ORG farming,[36–40] due to the fact that pesticides are not allowed. Thus the results of cytokines suggest the absence of possible contaminants in ORG carrots. These findings are in agreement with our previous study,[41] showing that in vulnerable conditions such as malnutrition the lymphocyte proliferative response was higher after ORG than CV wheat consumption, suggesting a lack of contaminants in ORG wheat that otherwise would have affected the proliferative capacity.

Carrots are among the major sources of carotenoids, which play an important role in the prevention of several diseases and in the modulation of the immune system.[9,10,42] However, it is still uncertain whether ORG carrots contain a higher carotenoid content than CV ones.[13–16] In our study the carotenoid content did not significantly differ between ORG and CV carrots within the same variety[43] (Paoletti *et al.*, submitted for publication), and therefore the ORG-mediated immune changes observed in our study cannot be ascribed to a difference in carotenoid content. Falcarinol is a prominent bioactive compound of carrots, which has been shown to reduce pro-inflammatory cytokine production.[11,12,44] Significantly higher levels of falcarinol were found in *Bolero* carrots of the second-year than first-year production, but no significant differences were observed between ORG and D-CV carrots of both years[45] (Edelenbos *et al.*, manuscript in preparation), in agreement with previous studies.[18] Thus, as for carotenoids, falcarinol likely did not play a role in the immune modulation by ORG carrots, and hence it remains to be established which carrot compounds may be responsible for the ORG-induced immunological stimulation.

CONCLUSIONS

The results presented in this study provide novel information on the health effects deriving from ORG food consumption, indicating

an immune stimulation by ORG carrots, especially by the 'more organic' O2 and O3 carrots, on both intestinal and peripheral immunity, including an expansion of Treg cells. In addition, this study reinforces the concept of the safety of ORG products. Finally, the results suggest that the analysis of an adequate number of harvests is advisable, owing to the possible great variability between different harvest years.

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