IN VITRO EFFECTS OF *ENTEROCOCCUS FAECALIS* AND SELECTED BIOMOLECULES ON THE MOTILITY OF RABBIT SPERMATOZOA

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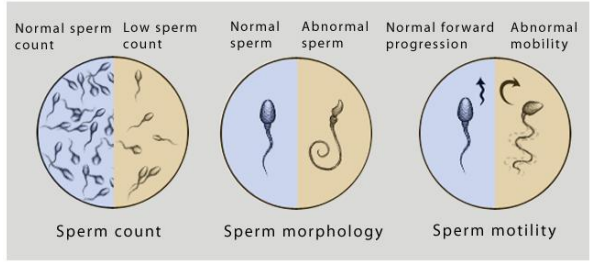
BACTERIAL INFECTION OF SEMEN

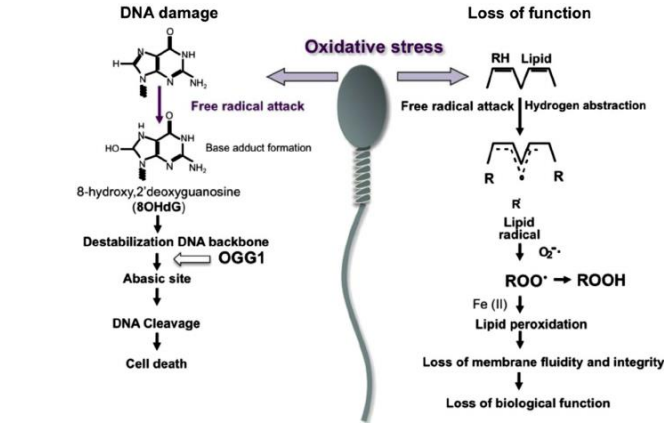
- Decreased sperm quality visible in routine semen analysis:
  - loss of sperm motility
  - morphological alterations
  - acrosome dysfunction
  - disruption of membrane integrity
  - oxidative stress

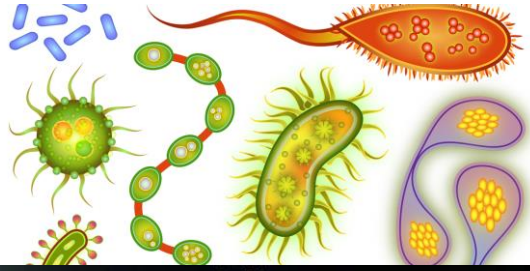
- Most data connected to bacterial contamination of ejaculates: well-known causative agents of urogenital tract infections
  - *Escherichia coli*, *Staphylococcus aureus*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Chlamydia trachomatis*

- Ejaculates collected for reproductive technologies - certain contamination:
  - semen collection is not an entirely serile process
  - factors for semen contamination: artifical vaginas, environmental conditions, human factors

- Current interest shifts to other bacteria, responsible for the colonization and contamination of the male urogenital tract, rather than infection

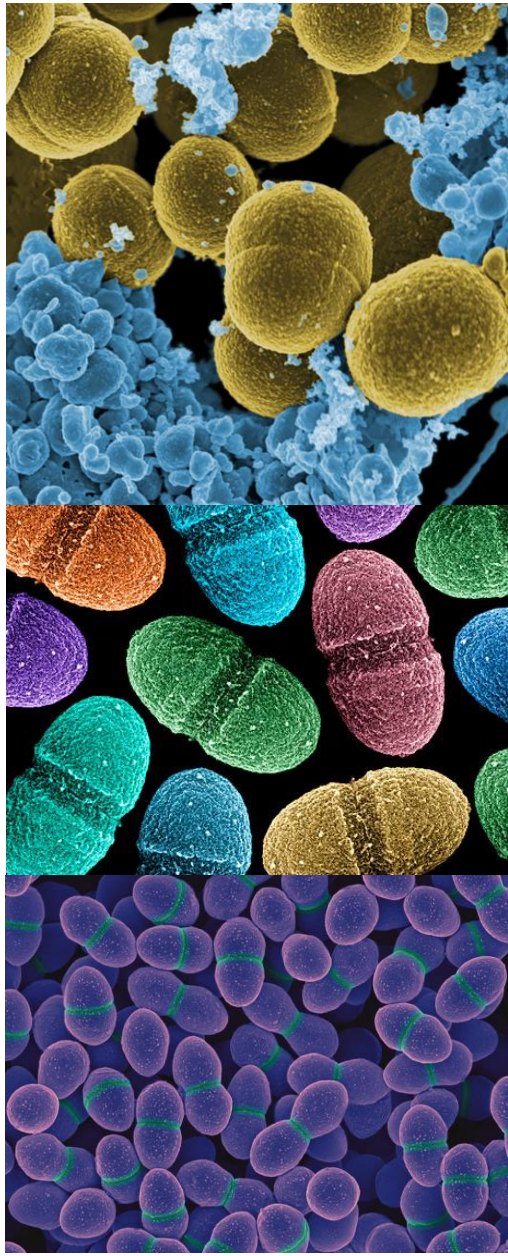






ENTEROCOCCUS SPECIES

- Gram-positive, catalase-negative, non-spore-forming, facultative anaerobic bacteria
- Lactic acid bacteria (LAB) that produce bacteriocins
- Origins: environmental, animal and human sources
- *E. faecalis:*
  - most common in the gastrointestinal tract, and may be found in human and animal faeces
  - associated with clinical urinary tract infections, hepatobiliary sepsis, endocarditis, surgical wound infection, bacteraemia and neonatal sepsis
  - able to survive a range of adverse environments allowing multiple routes of cross-contamination
  - resistant to a broad range of antibiotics including ampicillin, ciprofloxacin and imipenem



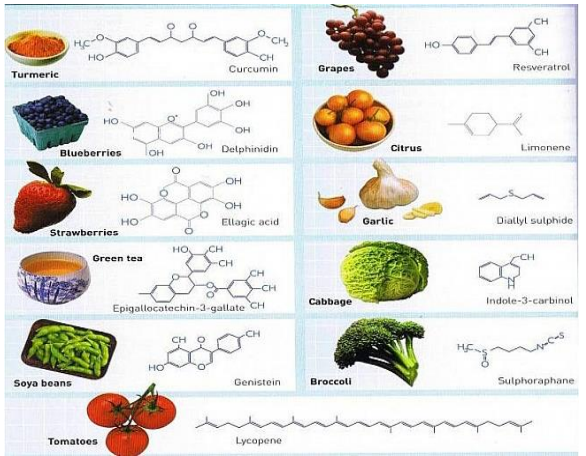
HOW TO AVOID SEMEN CONTAMINATION

ANTIBIOTICS

- Currently added to semen extenders to control bacterial contamination in semen arising during collection and processing
- May be toxic to spermatozoa
- Ever-increasing bacterial resistance
- An urgent need to find alternatives to conventional antibiotics for use in animal reproduction science

NATURALLY OCCURING COMPOUNDS

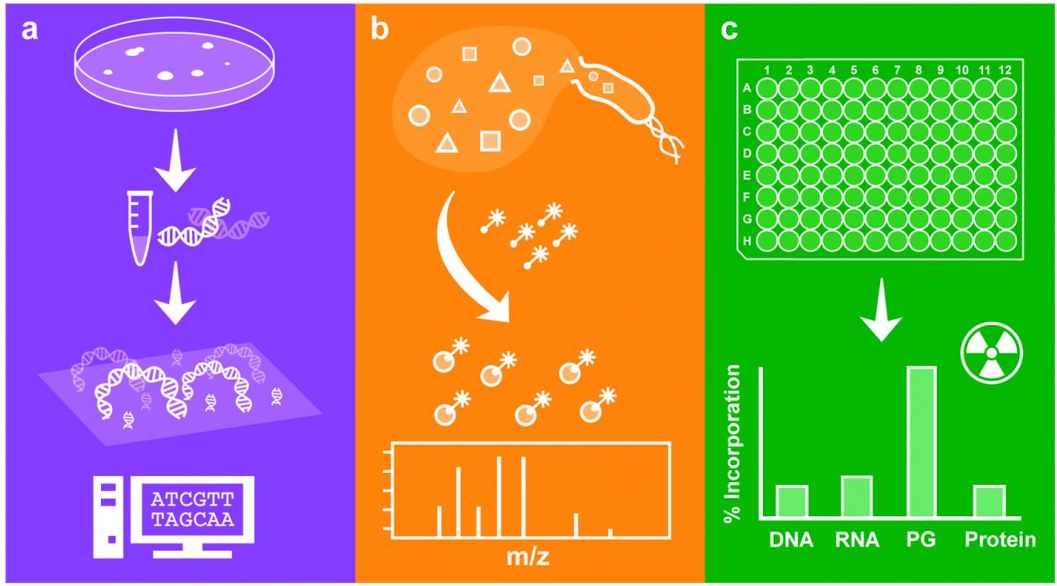
- Rich chemical diversity, structural complexity and availability, lack of significant toxic effects and intrinsic biologic activity
- Anti-inflammatory, antibacterial and antioxidant properties
- Selective advantage to male reproductive cells under stress conditions



AIM OF THE STUDY

- To assess the *in vitro* effects of:
  - Resveratrol
  - Quercetin
  - Curcumin
  - Epicatechin
  - Isoquercitrin

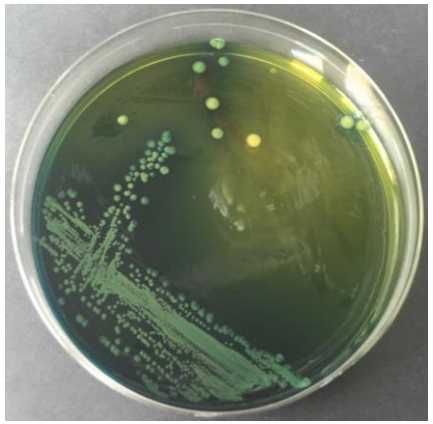
on the motility behavior of rabbit spermatozoa subjected to *in vitro* induced *E. faecalis* contamination

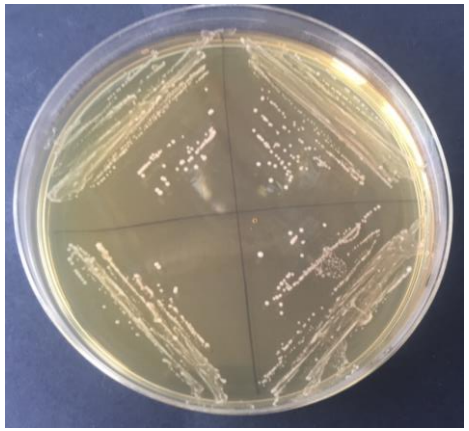


MATERIALS AND METHODS I.

Sample collection and identification of microorganisms

- Semen samples from 19 male New Zealand white broiler rabbits
- Assessment of sperm concentration and motility
- Sample transfer and culture:
  - MacConkey agar (37°C, 24h)
  - MRS agar (37°C, 48-72h)
- Purification of microorganisms: four ways streak plate method after the first cultivation:
  - chromogenic colifrom agar and URI Select IV to purify microorganisms from the MacConkey agar
  - repeated MRS agar purification





MATERIALS AND METHODS II.

Identification of microorganisms

- Matrix-assisted laser desorption/ionization time-of-light (MALDI TOF MS): bacterial identification in the semen samples
- Fresh overnight cultures: preparation of isolates
- Sample spot overlay : 2 µL matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid)
- Obtention of raw spectra: Biotyper software
- Transfer of the isolated *E. faecalis* to the culture medium selected for the *in vitro* experiments
- Cell culture at 36°C for 24 to 48h
- *E. faecalis* concentration adjusted to 0.3 McF
  - inoculum suitable to create an ideal environment for the sperm cells as well as the bacterium

Sample		Organism (most likely)	log score	Organism (2nd most likely)	log score
1	+	Pseudomonas oryzihabitans	1.85	Pseudomonas oryzihabitans	1.828
2	+++	Acinetobacter baumannii	2.375	Acinetobacter baumannii	2.283
3	+++	Acinetobacter baumannii	2.388	Acinetobacter baumannii	2.242
4	+	Pseudmonas sp.	1.728		1.410
5	+	Pseudomonas oryzihabitans	1.973	Pseudomonas oryzihabitans	1.849
6	+	Pseudomonas oryzihabitans	1.710		1.644
7	+++	Enterococcus faecalis	2.377	Enterococcus faecalis	2.334
8	++	Acinetobacter baumannii	2.250	Acinetobacter baumannii	2.153
9	+++	Acinetobacter baumannii	2.460	Acinetobacter baumannii	2.359
10	+++	Acinetobacter baumannii	2.406	Acinetobacter baumannii	2,313
11	+++	Enterococcus faecalis	2.441	Enterococcus faecalis	2.414
12	+++	Enterococcus faecalis	2.436	Enterococcus faecalis	2.427
13	+++	Enterococcus faecalis	2.485	Enterococcus faecalis	2.349
14	+++	Enterococcus faecalis	2.460	Enterococcus faecalis	2.379
15	+++	Enterococcus faecalis	2.481	Enterococcus faecalis	2.292
16	+++	Enterococcus faecalis	2.495	Enterococcus faecalis	2.349
17	+++	Enterococcus faecalis	2.468	Enterococcus faecalis	2.304
18	+++	Enterococcus faecalis	2.459	Enterococcus faecalis	2.293
19	+++	Enterococcus faecalis	2.442	Enterococcus faecalis	2.246

+++ highly probable species identification; ++ reliable identification of genus and probable species identification; + reliable identification of genus

MATERIALS AND METHODS III.

In vitro experiments

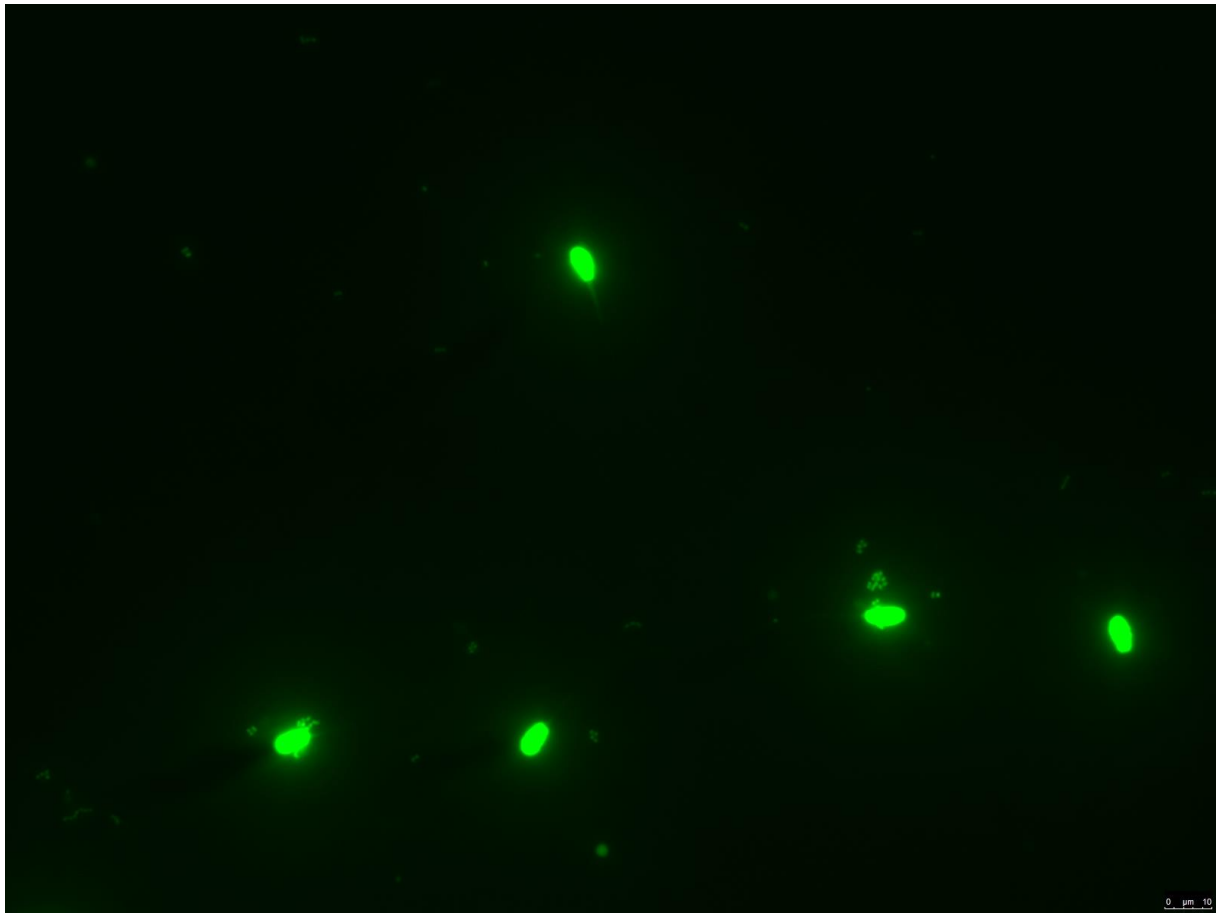
- 40 ejaculates from 10 male rabbits used for *in vivo* experiments
  - Minimum motility of 60%
  - Pooled samples
- Sample centrifugation, seminal plasma removal, sperm wash
- Sample resuspension in PBS + mineral supplements + 5% glucose + 4% BSA using a dilution ratio of 1:20
- Two controls:
  - Negative Control: culture medium exclusively
  - Positive Control: culture medium with 0,3 McF *E. faecalis*
- Experimental groups: exposure to the bacterium and different concentrations of chosen biomolecules:
  - 50, 10 and 5 µmol/L resveratrol (RES)
  - 50, 10 and 5 µmol/L quercetin (QUE)
  - 10, 5 and 1 µmol/L curcumin (CUR)
  - 100, 50 and 10 µmol/L epicatechin (EPI)
  - 100, 50 and 10 µmol/L isoquercitrin (IZO)
- Culture times: 0h, 2h, 4h, 6h and 8h
- Spermatozoa motility assessment:
  - computer-aided sperm analysis (CASA)
  - samples were stained using the IDENT stain
  - 10 microscopic fields were subjected to each analysis in order to include at least 300 cells

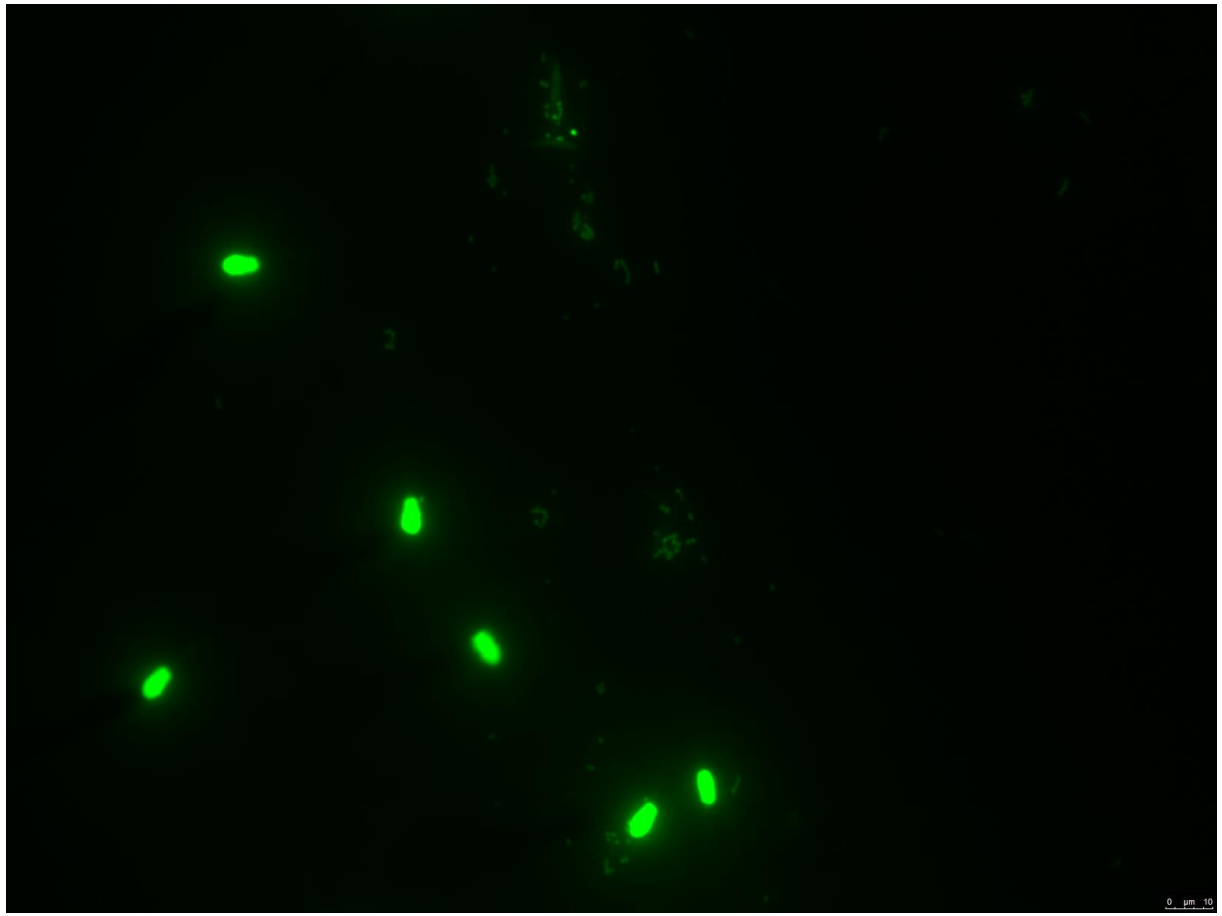
MATERIALS AND METHODS IV.

Statistical analysis

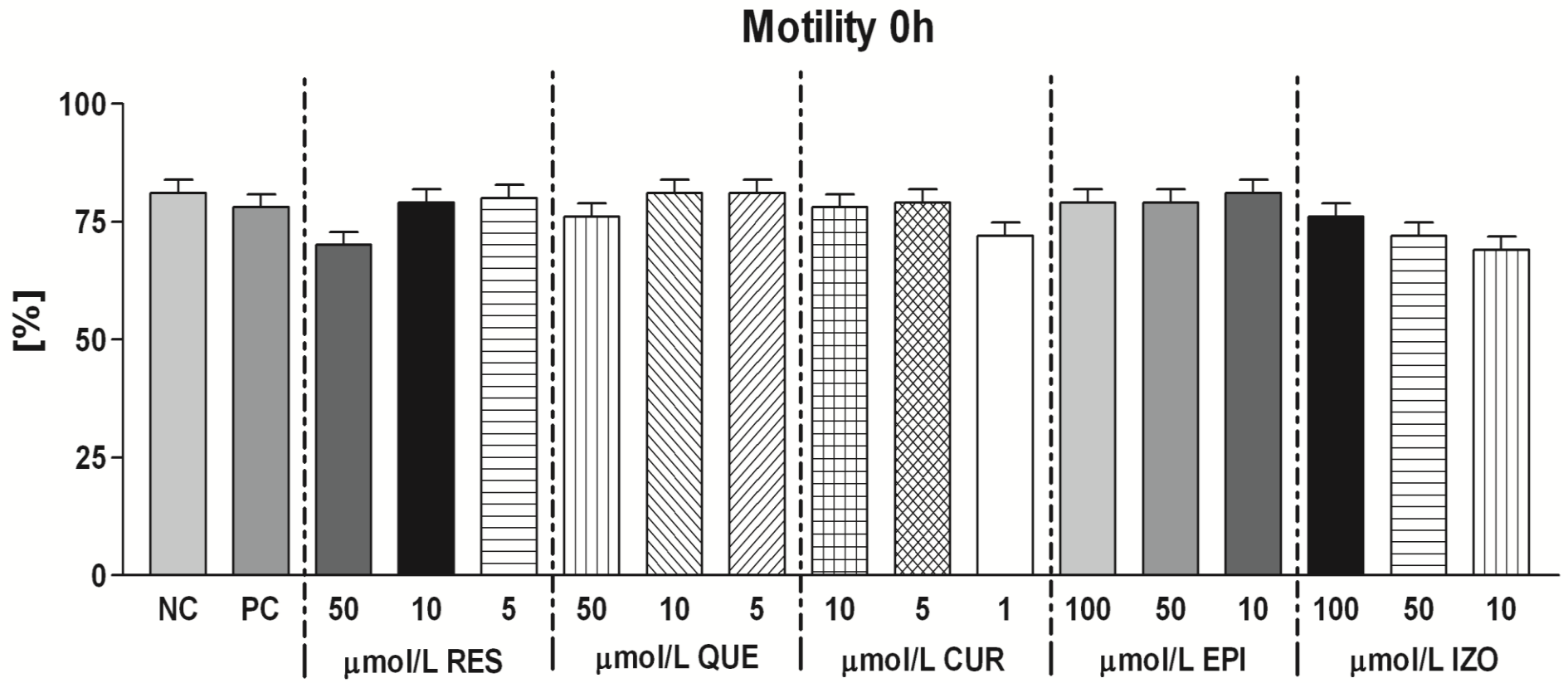
- GraphPad Prism program (3.02 version for Windows, GraphPad Software incorporated, San Diego, California, USA)
- Comparative analysis: one-way ANOVA with the Dunnett's post test
- Levels of significance: * $P<0.05$; ** $P<0.01$; *** $P<0.001$
- The comparative analysis was performed as follows:
  - Positive Control (PC) was compared to the Negative Control (NC)
  - Experimental fractions exposed to *E. faecalis* and biomolecules were compared to both Controls

RESULTS



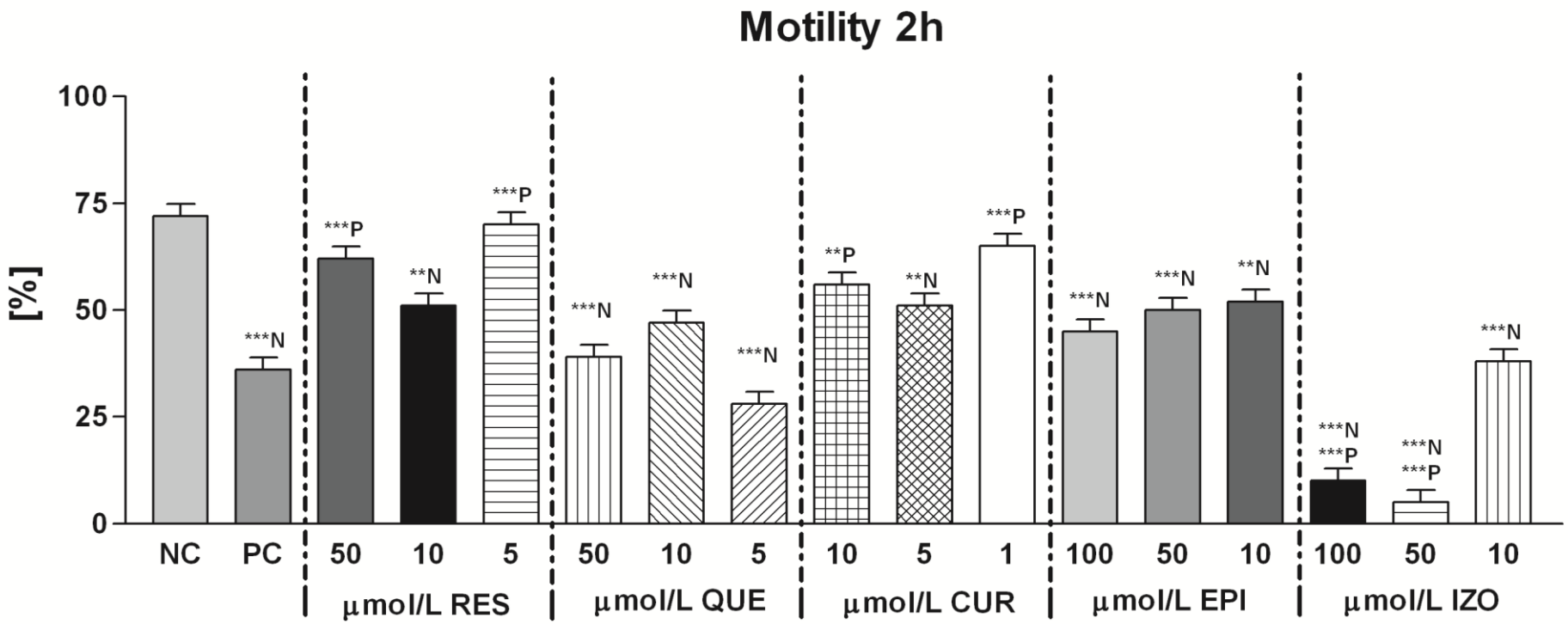


RESULTS I:
IMMEDIATE EFFECTS (TIME 0H) OF *E. FAECALIS* AND SELECTED BIOMOLECULES ON RABBIT SPERMATOZOA MOTILITY



MEAN ± SEM. * P<0.05; ** P<0.01; *** P<0.001

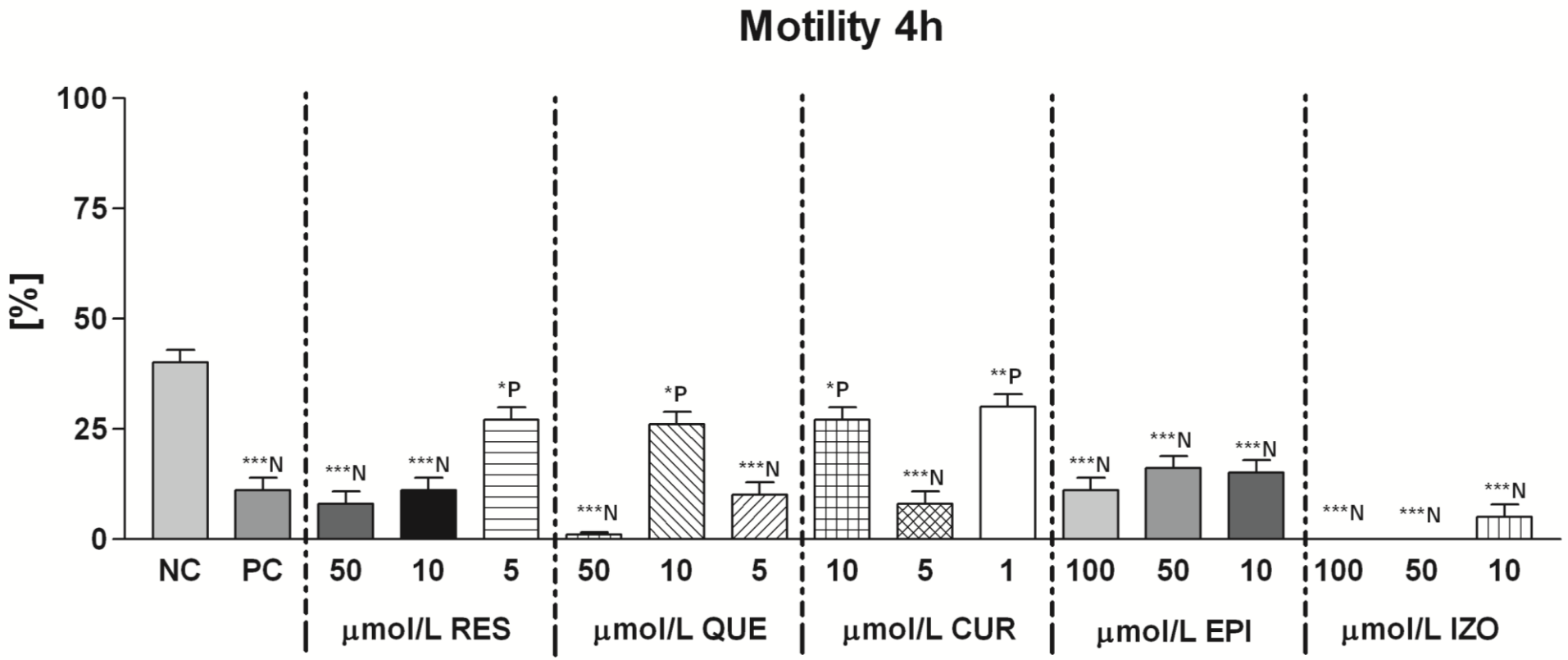
RESULTS II:
THE EFFECTS OF *E. FAECALIS* AND SELECTED BIOMOLECULES ON RABBIT SPERMATOZOA MOTILITY FOLLOWING 2 HOURS OF *IN VITRO* CULTURE



MEAN ± SEM. * P<0.05; ** P<0.01; *** P<0.001

N – VS. NEGATIVE (UNTREATED) CONTROL. P – VS. POSITIVE CONTROL (EXPOSED TO *E. FAECALIS* EXCLUSIVELY).

RESULTS III:
THE EFFECTS OF *E. FAECALIS* AND SELECTED BIOMOLECULES ON RABBIT SPERMATOZOA MOTILITY FOLLOWING 4 HOURS OF *IN VITRO* CULTURE



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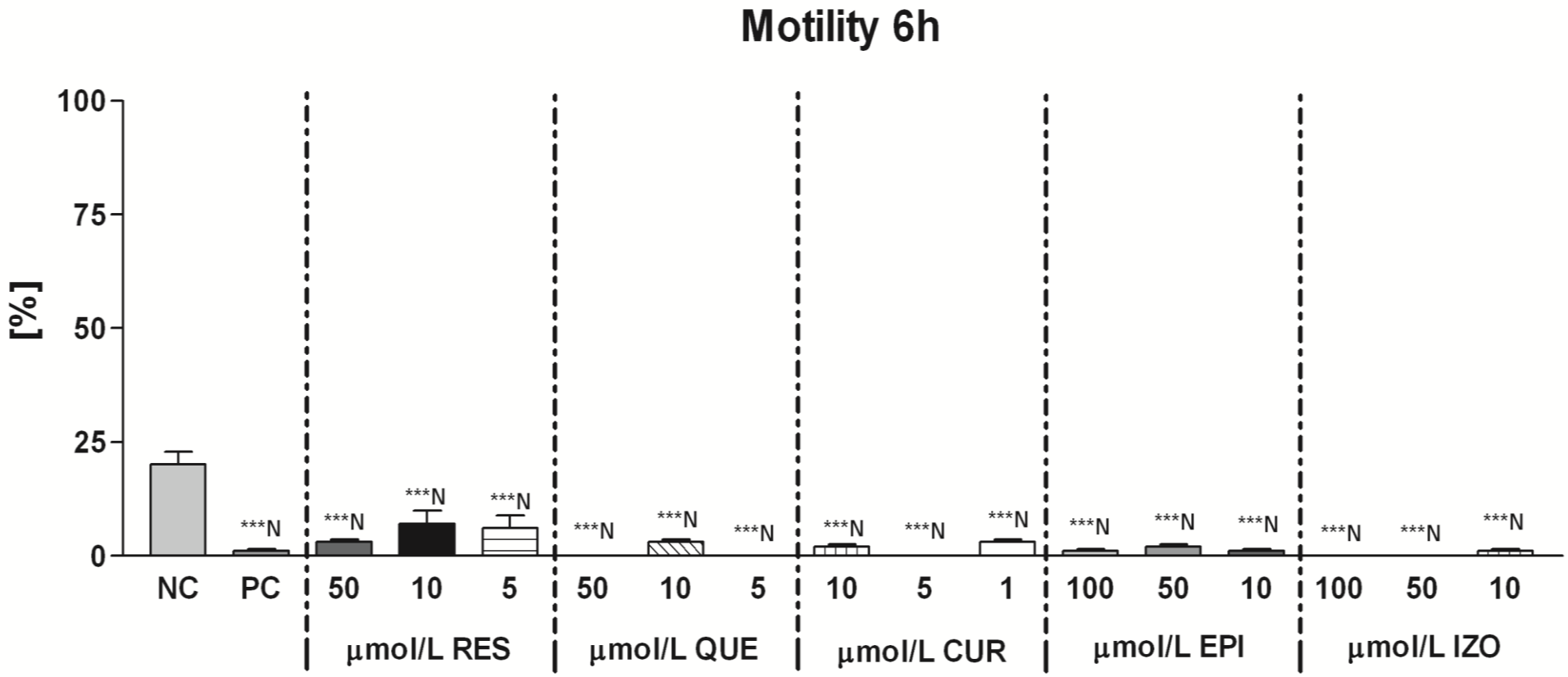
N – VS. NEGATIVE (UNTREATED) CONTROL. P – VS. POSITIVE CONTROL (EXPOSED TO *E. FAECALIS* EXCLUSIVELY)

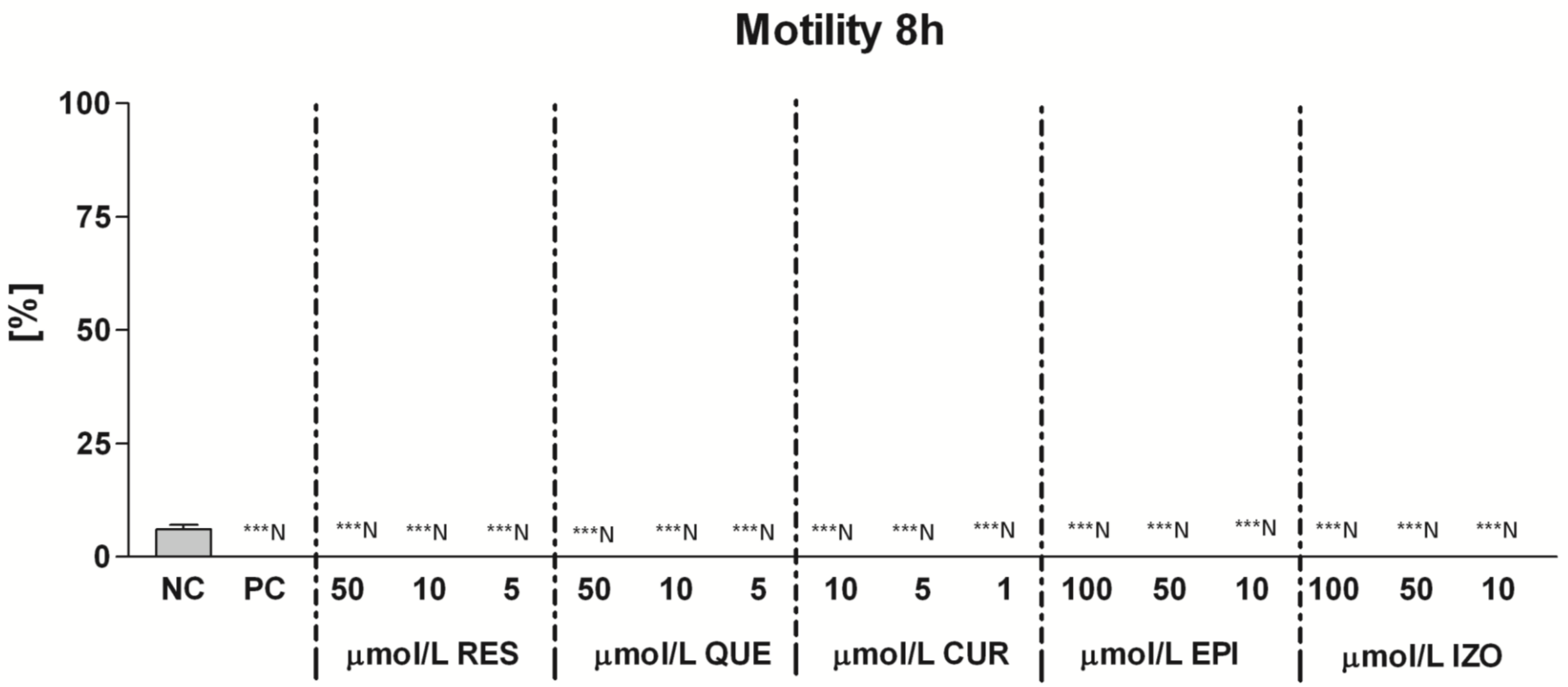
RESULTS IV:
THE EFFECTS OF *E. FAECALIS* AND SELECTED BIOMOLECULES ON RABBIT SPERMATOZOA MOTILITY FOLLOWING 6 HOURS OF *IN VITRO* CULTURE



MEAN ± SEM. * P<0.05; ** P<0.01; *** P<0.001

N – VS. NEGATIVE (UNTREATED) CONTROL. P – VS. POSITIVE CONTROL (EXPOSED TO *E. FAECALIS* EXCLUSIVELY)

RESULTS V:
THE EFFECTS OF *E. FAECALIS* AND SELECTED BIOMOLECULES ON RABBIT SPERMATOZOA MOTILITY FOLLOWING 8 HOURS OF *IN VITRO* CULTURE



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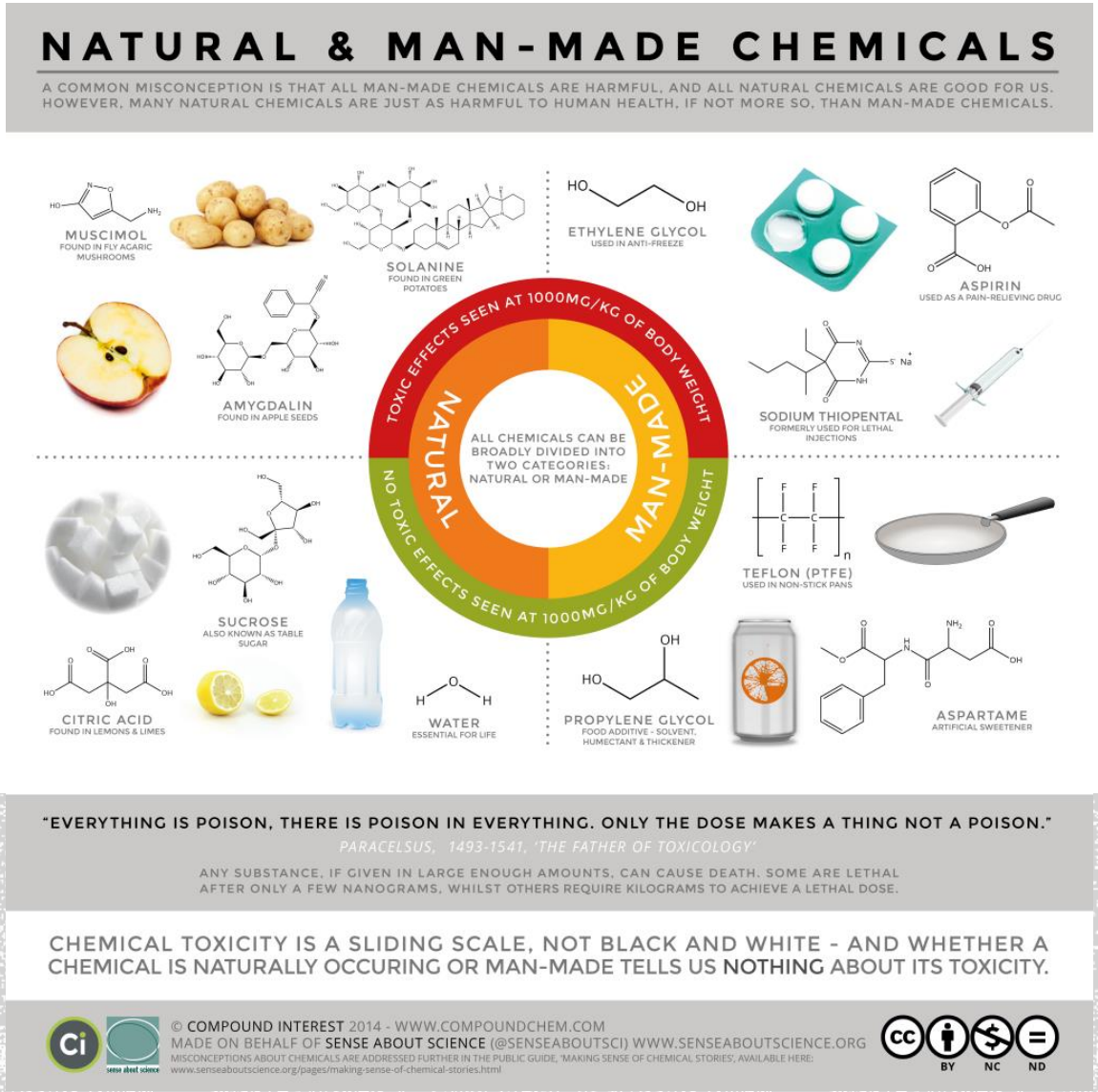
N – VS. NEGATIVE (UNTREATED) CONTROL. P – VS. POSITIVE CONTROL (EXPOSED TO *E. FAECALIS* EXCLUSIVELY)

CONCLUSIONS

- Resveratrol, quercetin and curcumin exhibit antibacterial properties:
  - provision of a selective advantage to the male gametes in the presence of *Enterococcus faecalis*
  - particularly during short-term rabbit semen handling
- Epicatechin and isoquercitrin did not prove to possess significant protective or beneficial effects on the *in vitro* survival of rabbit spermatozoa in the presence of *Enterococcus faecalis*
- More experiments will be necessary to unravel specific molecular mechanisms of action of *E. faecalis* and/or natural biomolecules on the structure and function of male reproductive cells

THANK YOU FOR YOUR ATTENTION



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